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LECTURE NOTES IN MEDICAL PROTOZOOLOGY

BY

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PREFACE

A word of preface to this little volume may perhaps be permissible to explain the circumstances under which it came to be written. When the writer first began to teach medical protozoology to post-graduate students at the Calcutta School of Tropical Medicine in October 1921 he found himself faced by considerable difficulties. The subject cannot be properly taught unless it is profusely illustrated. Hence resort was had to the very complete library which Sir Leonard Rogers had already collected as a nucleus for future developments. From some hundred different books and journals, plates and illustrations were selected, as far as possible the original ones of the authors concerned. With the assistance of Mr. H. Heins of Messrs. Houghton, Butcher, Eastern, who possesses apparatus for the purpose of exceptional clearness of definition, and who is probably the best lantern slide expert in India, a collection of some 450 lantern slides was prepared, to be subsequently kept up to date, and supplemented by cinema films of motile protozoa and of protozoal life cycles from Messrs. Pathé Frères, and by the very free use of the epidiascope. A Parallax electric lantern with a 1000 c.p. arc was installed and fitted with a very exceptional Dallmeyer projection lens : the result being pictures eight feet square on the screen with perfect illumination and the utmost clearness of detail.

The difficulty of illustrating the lectures was now solved, but a further difficulty presented itself in consequence. The students at the School are all post-graduates, and are, most of them, picked and selected medical practitioners from a host of applicants. They pay a considerable fee for the course, and they are men who are very keen and willing to learn. To teach such students is very different from the uninteresting round of trying to bring medical undergraduates up to the scratch for a pass degree. Yet, as arranged, the lectures go through in darkness, with lantern, cinema and epidiascope working, and it is essential to try and capture the student's entire attention to what is being said and to the screen. Also the darkness militates against note taking.

Further, although the students, all of whom are qualified and mostly practising medical men, English, Anglo-Indian and Indian, have some smattering of clinical medicine and of the rudiments of pathology, the fascinating world of the protozoa is to them an absolutely sealed book. It would not be fair to the subject to teach such men the parasitic protozoa of man, without attempting to shew them how such protozoa fit in as a mere section of the greater world of the parasitic protozoa in general, and of the whole immense domain of the general protozoa. Systematic teaching is here of the utmost importance if the student is to grasp even the essentials of the subject. Also accurate teaching is even more important. Thus the difference between a gametocyte and a gamete may seem to the writers of text books on tropical medicine a mere trifling with words : yet the hypothesis has been seriously put forward that relapses in malaria may be due to conjugation of gametocytes within the same erythrocyte in the human blood stream. A knowledge of the elements of protozoology would have made such a suggestion obviously untenable : what conjugate are not gametocytes but the gametes which are their offspring, and—further—the gametes of the *Haemosporidia* do not conjugate within the body of their vertebrate, but in the gut or tissues of their invertebrate hosts. Schaudinn, distinguished protozoologist as he was, once suggested that relapse in malaria might be due to parthenogenesis on the part of malarial macrogametocytes, and this erroneous view dominated protozoology for many years. Had he paused to think a little more accurately he would have realised that the progeny of a parthenogenetic macrogametocyte should not be the merozoites which he figured, but sporozoites. It is impossible to over-emphasise the importance of accurate and not slip-shod teaching. The students at the School will, many of them, become the teachers of medicine in the new India of tomorrow : they must be given of the best.

The writer, therefore, set about the production each week of very full and detailed type stencilled precis lecture notes. These delivered the student from the necessity of note taking during the lectures. Yet they proved far from satisfactory. Often the ink or paper was bad,—in fact in such materials India appears to import all the rubbish that Europe has no use for : the cost was not inconsiderable ; and although duly grateful, the students were not satisfied. The alternative was to go into print. Here there were two possibilities. Either a new text book of medical protozoology could be written, or the lecture notes revised, brought up to date, and printed.

The first alternative proved impossible. There is today a very urgent need of such a text book. The science of protozoology makes such incessant strides that almost every book is out of date shortly after it is issued. Dobell and O'Connor's splendid monograph on the intestinal protozoa of man covers one section of the subject, but one only. The literature on the ***Binucleata*** and on the ***Haemosporidia*** is scattered through the medical research journals of the past twelve years : that on the spirochaetes is ever expanding. There exists today, as far as the writer knows, no one single text book on the subject in English which is in any way adequate, and which can be placed in the hands of students as a sure and reliable guide. Yet such a book would take a year at least to compile, it would have to be perforce profusely illustrated, it would probably not pay any publisher to produce it since the demand for it would probably be limited. Last, but not least, when once produced its price would probably be so high as to place it beyond the pockets of the particular students for whom it was intended. Patton and Cragg's splendid Entomology is a classic, but it is rarely found except in libraries. The Indian Research Fund Association in 1922 very kindly considered the possibility of financing such a text book, but at the eleventh hour the descent of the Inchcape " axe " rendered any assistance from this direction improbable.

The second alternative has accordingly been adopted. It is hoped that in this volume the student is presented with such notes as a competent shorthand typist, conversant with the subject, would take, and with a very full precis of each lecture. Every attempt has been made to make the volume complete with practical notes of value and with full technique for laboratory methods. The most important literature has been added for reference at the end of most lectures. The writer hopes that here, in small bulk and in convenient form, is presented to the student the essentials, both theoretical and practical, of medical protozoology.

The publication of this volume has been rendered possible through the very great kindness and generosity of the Hon'ble Sir Rajendra Nath Mukerjee, K.C.I.E., who has very kindly paid for its publication. Sir Rajendra Nath Mukerjee has made many and notable donations to the Calcutta School of Tropical Medicine, and the School owes much to his personal generosity and even more to his labours on behalf of it and to his support on the Governing Body of the Endowment Fund of the School in the financing and support of medical research work in Calcutta and in India. The present departure however is novel : it represents a beginning of the endowment of post-graduate medical teaching in India. Thanks to Sir R. N. Mukerjee's generosity this little volume has been published.

It will be sold at a slight profit and all proceeds will be vested under the Governing Body of the Endowment Fund of the School for the purpose of creating a small and subsidiary fund for the production and publication of text books, lecture memoranda etc.,—even, possibly, some day of a medical research journal in connection with the School.

Thus the present venture is but preliminary, and in no way final. This edition has been limited to 500 copies. The chief sale will probably be to students of the School, but the writer hopes that—possibly—the book may prove of interest to others. All income from its sale will be credited to the Endowment Fund of the School. As soon as the initial cost of publication is recouped, and funds are again available—possibly in 12 to 18 months' time—a second edition will be brought out, if required, brought fully up to date and incorporating the ever growing volume of incessant research work upon the subject. It is thus hoped that the book will be brought out for the benefit of the students concerned at frequent intervals, in each case re-written and containing all the latest information available.

The writer regrets that medical protozoology cannot be taught without trespassing upon controversial problems. He may have made many errors and have fallen into many unconscious blunders in this volume. He will be extremely grateful if such errors are pointed out to him. It is almost impossible for any one isolated worker to keep abreast of a subject of such dominant importance in the ever widening field of tropical medicine. If the writer has succeeded in placing in the hands of his students a detailed precis of the subject he will be content: should this small volume command a wider sale and interest he will be surprised and gratified.

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CENTRAL AVENUE, CALCUTTA.
1st May, 1928.

POSTSCRIPT.

The delay in the publication of these notes, which have been for nearly four months in the press, is very much regretted. Whilst it was in press it was found that the ultimate cost of printing would be very considerably in excess of the original estimate, and the writer approached Sir Kailas Chandra Basu, Kt., C.I.E., whose devoted labours on behalf of the School are well known. Sir Kailas responded by taking a personal interest in the project, and Mr. Ganshyam Das Birla came forward with a very generous contribution of Rs. 500 to the proposed Book Fund, a donation which is but one of his several generous donations and bequests to the School. The Book Fund is thus really placed upon its feet, and will, it is hoped, become a useful feature of the work at the School.

There are several minor points in this book with which even the writer himself is dissatisfied. It ought, of course, to be profusely illustrated and indexed, but both procedures would have added materially to its cost. Names of genera and species ought throughout, of course, to be in italics, but this would lead to such incessant changes of type as to have again increased expense. Only two proofs have been submitted to the author, and, through an unfortunate mistake, galley proofs only of the first 32 pages : proof reading has had to be done hurriedly and amid the pressure of other work, and several obvious mistakes in spelling have remained undetected. The writer had intended to devote the leisure of the vacation to proof reading and correction, but unfortunately the book was only half ready when the current session started and its final publication has been hurried in consequence. At the same time the writer's most grateful thanks are due to the Banerjee Press for the very great trouble to which they have gone with regard to it.

1st September, 1928.

R. K.

LECTURE I.

ORIGIN AND CLASSIFICATION OF THE PROTOZOA.

Immense range of protozoa, wherever air and moisture. Importance in biology, zoology, cytology, embryology, parasitology and medicine. Immense world of protozoa, free living, saprophytic, harmless commensals, and truly parasitic. All animal and even plant life parasitised by protozoa.

Protozoa formerly defined as single celled animals : better is Dobell's definition as non-cellular animals. Some groups shew colony formation : but colony is simply aggregation of similar individuals. Animal kingdom divided into Protozoa and Metazoa : protozoa of equal importance with whole of metazoa. Derivation of term protozoa from protos=primitive and zoon=living animal. Cell theory of structure introduced by Schwann, 1838 : created modern histology and pathology. Definition of cell as structural unit of life. Such protozoa as more complex Ciliophora have almost equal complexity with fully developed mammals, but whole contained in one non-cellular individual.

Age of earth at least 1,000 million, perhaps 2,000 million years. Earth habitable to life for at least 100, perhaps for 800 million years : e.g., fossils in Cambrian strata of 376 million years ago (?). Beginning of life on globe. Arrhenius' view of arrival of ultra-minute spore-like particle from space, driven by pressure of radiant light. Other views as to origin amid welter of chemical complexes occurring on surface of still hot and cooling earth crust. Immediately life principle appeared, says Bergsen, two dominant principles arose in Nature : inanimate matter streaming down a vortex of disintegration, breaking down into simpler elements, with liberation of immense stores of radio-activity. Life, on the other hand, commencing from ultra-minute origins, ascending up ever widening tree of evolution and progress. Evolution of instinct along one line, (with organs adapted for new functions) : on other of intelligence, utilizing external tools : the "élan vital." The amoeba to man perhaps only 100 million years, but a story of continuous evolutionary adaptation and progress.

Protoplasm semi-fluid jelly : translucent, colourless. Chemical constitution see text books. Impossible to see much structure in such transparent objects : hence use of fixatives and stained preparations. Fixatives used to kill protozoa in situ, coagulate cell protoplasm, fix cell structure. Formalin bad fixative. Best are Schaudinn's and Zenker's : for formulæ see Practical Notes. Stained preparations may not exactly correspond with living structure, but represent fixation images. Views as to structure of protoplasm : Butschli's foam theory : mixture of drops of liquid with denser substance in alveolar walls between, like foam of oil, water and soap. Radio-active jellies simulating protoplasm.

Primordial protist organism. Respiration by osmosis. Food supply at first by osmosis, also excretion of waste products. Balanced metabolism. Later amoeboid movement in search of food stuff and ingestion of food particles. Amoeboid movement by formation, extrusion and withdrawal of pseudopodia. Chemotaxis, positive and negative : phototaxis, thermotaxis. Feeding now by osmosis plus ingestion. Formation of food vacuole around ingested food particle : digestive juices of protozoa. Excretion of waste products : formation of contractile vacuole. Amoeba leaving trail of waste matter as it moves.

Internal streaming circulation of protoplasm inside protozoal cell. Development of nucleoplasm : differentiation of cell protoplasm into nucleoplasm and cytoplasm. Nuclear chromatin with intense affinity for certain dyes : jet black with Iron hæmatoxylin : ruby red with all Romanowsky stains, e.g. Leishman's. Idiochromidia : chromatin present as innumerable fine grains throughout cytoplasm. Concentration of chromatin into karyosomes, into nucleoli, into nuclei. All stages seen in protozoa : Arcella shews idiochromidia plus several fusion nuclei. Binucleate protozoa : with different functions of micro- and macro-nuclei, e.g., trypanosomes and Leishmania genus. Different nuclear patterns in protozoa : nuclear reticulum of non-staining achromatin=linin network. Chromatin threaded on linin network as grains, granules, bars, lumps, etc. Evolution finally of metazoal nucleus : cf. tennis ball, fill with jelly, insert coiled up string of beads. Then jelly=nuclear sap : string=linin network : beads=chromatin : ball=nuclear membrane. Aggregated knots of chromatin called karyosomes, plasmosomes, nucleoli.

Cell now contained cytoplasm and nucleus. As it grew in size need arose for division. Volume of sphere proportional to cube of its radius : hence after reaching certain size protozoon had to divide in order to keep all parts of body nourished. In metazoal cell is tiny spot just outside nuclear membrane= centre for cell division or centrosome. Around it is archoplasmic vesicle. Originates from nucleoplasm: in many protozoa is intranuclear. Where centrosome governs movements of protozoon it=and is called a blepharoplast. When cell divides first thing is division of centrosome and archoplasmic vesicle.

Two types of cell division. Amitosis or direct division very simple : division in turn into two of nucleus, cytoplasm, cell membrane.

Mitosis or karyokinesis more complicated. See text books for plates etc. Linin network becomes regularly arranged as spireme. Chromatin becomes divided up into regular chromosomes arranged on linin spireme. Number of chromosomes same for each species, e.g. 32 for guinea pig and man, 2 for some species of Ascaris. Next longitudinal division of linin spireme along its whole length : and of attached chromosomes, whose number is now doubled. Nuclear membrane disappears. Centrosomes move to opposite poles of cell : between them formation from nucleoplasm of outer set of mantle fibres (like lines of longitude around globe) with chromosomes attached to them : inner set with no attached chromosomes running through from pole to pole=centrodesmose. Chromosomes arranged around equator of cell=equatorial plate. Next mantle fibres contract towards two poles of cell, drawing chromosomes after them. Half total number pass to each pole : hence new cell has usual number of chromosomes for species. At each pole linin becomes again spireme : new nuclear membrane formed : centrosome moves out of nucleus into cytoplasm : new nucleus at each pole. Finally division of cytoplasm and cell membrane.

Cell division in protozoa of all types : varying from amitosis to true karyokinesis and shewing many intermediate variations. E.g. Dobell shews in binary fission of Entamœba histolytica formation of division spindle, and Kofoid claims definite and different number of chromosomes in E. coli and E. histolytica. (?).

Structure of protozoa infinitely varied from simple Amœba to highly complex Ciliophoron. All approximate more or less towards typical adult metazoal cell. Structure of typical cell : see any text book. Cell membrane (absent in some protozoa, as amœbæ): cytoplasm with food granules, food vacuoles, water vacuoles, excretory and contractile vacuoles, foreign bodies, bacteria, yeasts etc., e.g. plastids or food reserve stores elaborated by nucleoplasm but laid down in the cytoplasm as food reserve, e.g. glycogen in cysts of E. coli and the chromatoid bars in cysts of E. histolytica. Pigment in some protozoa. Centrosome with attraction sphere=Golgi apparatus, governing cell movements and cell division. Nucleus with nuclear membrane, nuclear sap, linin network, chromatin, karyosomes, nucleoli etc.

Thus from primitive protist evolved by degrees fully developed metazoal type of cell. Protozoal forms of all intermediate types still exist today. Usual type of protozoal division is by simple binary fission into two : but often by multiple fissuion into cluster of daughter individuals, e.g. malarial rosette. Other types of reproduction, as will be seen later : multiplication within a cyst : sexual reproduction etc.

Hæckel's kingdom of the protists the primitive procursors of all life on globe. At early phase divided into two kingdoms : plants which are mainly sedentary, have chlorophyll, and breathe in CO_2 : and animals usually motile, with no chlorophyll, breathe in oxygen and often ingest food particles. Interesting problems as to whether any direct descendants of the primitive protists still exist, and are neither plants nor animals, but intermediate ?? do the Spirochætes, e.g. belong to such a group ?? Bacteria are plants.

Protozoa the result of 100 or more million years of evolution and adaptation to ever changing environment. Hence their extraordinary beauty and complexity. Thousands of dead and gone genera and species. Present day protozoa are the survivors of evolution : scanty pages of torn and tattered book. Hence difficulty of proper classification. (N.B. Avoid Castellani and Chalmers : hopeless classification and nomenclature). Usual classification into four big, main Phyla.

I. Sub-Kingdom Protozoa. Phylum Sarcodina (from Sarcos=flesh). No cell membrane, cytoplasm naked, amœboid movement by pseudopodia, hence constantly changing shape : multiply by fission and cyst formation. High types (Radiolaria) have calcareous skeletons deposited on surface.

Examples : free living amœbæ : parasitic entramœbæ : Heliozoa.

II. Sub-Kingdom Protozoa. Phylum Mastigophora (from mastix=a whip). Possess one or more whip-like motile organs known as flagella, for locomotion and food capture. Have cell wall and definite body shape. Multiply by binary and multiple fission, cyst formation, sexual reproduction. Shew special tendency to colony formation: e.g. L. donovani. Include intestinal flagellates, parasites of kala azar and oriental sore, trypanosomes: innumerable free living forms in earth and water: innumerable parasitic forms in plants, insects, reptiles etc.

III. Sub-Kingdom Protozoa. Phylum Sporozoa. All are parasitic. Inhabit blood or tissues or gut of man, animals, birds, reptiles, insects, crustaceae, fishes and even plants. No specialised organs for food capture or locomotion (not needed). Immensely important phylum in medicine. Reproduce by spore formation, whence name. Include Gregarines, Coccidia of man and animals and birds, Piroplasmata, Hæmogregarines, malarial parasites of man, Plasmodia of apes and birds, etc. Those Sporozoa which inhabit the blood are separately classed as the Hæmosporidia: and they have two life cycles, one in the vertebrate host, where they multiply asexually by schizogony: the other in a transmitting invertebrate host, in which they multiply by sexual reproduction, sporogony. E.g. malaria and the mosquito.

IV. Sub-Kingdom Protozoa. Phylum Ciliophora: (or often termed the Infusoria or Ciliata). Very complex and highly organised protozoa. Move by a coating of fine, hair like, vibratile organs known as cilia, whence name of group. Definite cell membrane and body shape. Reproduce by binary fission, encystment and sexual reproduction. Possess from two to many nuclei. Have special cell mouth or cytostome (in most species). Majority are free living forms especially seen in pond water, sewage etc.: but some are parasitic. Examples:—Paramœcium of dung water, innumerable free living forms such as Stylonychia, infusoria of ponds, infusorian parasites of rectum of frogs, toads, cockroaches: Balantidium coli of man and pig.

In brief:—1. Amœboid protozoa, many free living, many parasitic. 2. Flagellate protozoa, many free living, many parasitic. 3. Sporozoa, all parasitic. 4. Ciliate protozoa, many free living, some parasitic.

Also included in lecture course:—5. Spirochætes: doubtful whether these are protozoa or not: probably not: and 6. Chlamydozoa (Chlamyx=a veil), very doubtful whether these are really living forms or not: many are certainly not living forms at all but are lifeless cell inclusions: e.g. Negri body of rabies, Kurloff body of guinea pig.

Practical Class.

Study of methods of movement of Protozoa. Use the microscope set vertically: tube length out, concave mirror, condenser racked well down, iris partly closed. At all costs get critical illumination. This is the most important point of all in examining unstained protozoa.

To slow down movements add solution of gum, or tiny drop of serum. To study feeding add tiny drop of Indian ink. To see flagella and cilia and bring out nuclei mount under cover slip in solution :—Iodine 1 gm., Potass. Iodide 2 gms., water 100 c.c.

To kill motile protozoa in situ : make a thin film of the fluld on a slide. Invert it upside down over a wide mouthed jar containing :—Osmic acid 1 gm., Chromic acid 2 gms., water 100 c.c. Cover with paper or Petri dish etc. to keep fumes in. The osmo-chromic vapour kills and fixes the motile protozoon with flagella, cilia etc. still protruded. Expose for two minutes (or a little less : not more). The slide can now be allowed to almost dry before using the fixative. Expose a drop in this way over Osmo-Chromic acid : then add the iodine solution and the flagella and cilia will shew up clearly.

Donaldson recommends the following method to shew flagella and cilia. Mix freshly equal parts of (a) saline saturated with eosin and (b) 5% Potass. iodide in saline saturated with iodine. After killing the protozoa over osmo-chromic vapour to get flagella extended, add above mixture to emulsion. The flagella or cilia usually stand out clearly.

Fixatives.

1. For blood films. The simplest and one usually used is methyl alcohol. Pour on to the slide till it is covered : leave on for a minute : wash off with water. The film is then fixed and ready for staining. Leishman's stain is made up with methyl alcohol , so no preliminary fixaton is necessary.

Or :--(b) absolute alcohol. Pour on, and leave about ten minutes. Then wash off with water.

2. Fæcal films. The only fixative which will give a good cell picture is Schaudinn's. Saturated solution of corrosive sublimate in normal saline 2 parts : absolute alcohol 1 part : acetic acid (glacial) 5 c.c. per 100 of above mixture. Is best used warmed to 60°C on a water bath. Just before the film dries, and whilst it is still moist place very gently into the fixative : leave 10 minutes. Or if used cold, leave 20 minutes.

After using Schaudinn's or any fixative containing mercury it is next necessary to get the mercury out of the film. Remove the slide from Schaudinn's fluid. Wash well under the tap. Soak the film with 70% alcohol tinged a deep brown with Iodine. The film should now turn a deep brown : the mercury changing to mercuric iodide. This must next be dissolved out : soak the film with rectified spirit till all brown is washed out=mercuric iodide dissolved out. Transfer (best via graduated alcohols) to tap water : wash thoroughly : transfer to distilled water.

To get good preparations of motile protozoa it is best to expose the film for a minute to osmo-chromic vapour : leave till just before it commences to dry : then fix as above with Schaudinn's fluid.

3. For tissues—always—Zenker's fluid. Remove the tissue direct from the body and drop it into the Zenker. Use ten parts of Zenker to one of tissue. After 48 hours transfer to 70% alcohol.

Zenker's fluid is Potass. bichromate 1.5 gms., Sod. sulphate 1 gm., Hydrarg. Perchloride 5 gms. Water 100 c.c. Add 5 c.c. glacial acetic acid to each 100 c.c. just before use.

NOTE.

For a general review of the biology of the Protozoa the student cannot do better than to read :—

Minchin. Introduction to the Protozoa. Chapters 1 to 7 and especially Chapter 10.

The fist, 1912, edition of Minchin is better than the 1917 impression : which is merely a reprint on worse paper.

LECTURE II.

SARCODINA, AMŒBÆ, ENTAMŒBÆ, ENDOLIMAX NANA OF MAN.

Sarcodina divided into :—1. Rhizopoda and 2. Actinopoda. Latter are free living, often marine forms, with deposited lime skeleton and fine, ray-like pseudopodia streaming through them. Former, 1, alone concern us. Are creeping Sarcodina with branching or root-like pseudopodia. Only Order of them of importance to us are the Amœbæ.

Amœbæ universal wherever air and moisture. Innumerable free living forms in ponds, on decaying trees and vegetable matter, cabbage infusion, streams etc. A. limax e.g., very common : has much ectoplasm, and when moving streams into ribbon shape. A. verrucosa has ectoplasm toughened and often sandy particles adherent to it. A. proteus with branched, finger-like pseudopodia very common object in pond life. Good example of free living amœba. Has little clear ectoplasm. (Ectoplasm of A. limax on other hand very marked). Granular, voluminous endoplasm, stuffed with food particles, granules, vacuoles, debris. Breathes by osmosis. Moves by amœboid movement by pseudopodia. Feeds partly by osmosis, partly by ingesting solid food particles. Excretes by osmosis : also by contractile vacuole. Multiplies by binary—also possibly multiple fission. When reaction unfavourable, e.g. pond dries up, it encysts. Cysts are hardy, resistant forms : survive complete desiccation for a month or more. Tide over time till conditions favourable again. Important to know process of encystment of amœba. Motile amœba becomes motionless, pseudopodia withdrawn, rounds up, becomes little, clear ball. All food particles, debris etc. thrown out Endoplasm becomes glassy clear. Secretes thick, protective, tough cyst wall around it. Nucleus either remains single : or may multiply into 2, 4, 8, 16 etc., daughter nuclei. When conditions favourable again : cyst wall ruptures, around each nucleus aggregates cytoplasm : out of cyst creep from 1 to 16 little amœbulæ : start life again as new motile individuals. Thus we have two life cycles: 1, when conditions favourable multiplies, whilst still motile and in vegetative state, by schizogony: when conditions unfavourable forms cyst and multiplies within it = sporogony (spore formation). This the rule with all amœbæ and entamœbæ.

Any number of free living amœbæ: classification of species still very confused. Many have quite complicated life cycles. Arcella, e.g. is one of the shelled amœbæ. Begins life in spring as tiny, naked amœbula. Becomes bean shapped and secretes chitinous wall, with a pore in it, through which pseudopodia are protruded. Has no less than six different types of multiplication (vide diagram Minchin. 1912 edition, fig. 80). Binary fission, cycle D, E, F, G, D. Two nuclei and idiochromidia divide into two. 2. Exogenous budding, buds thrown out: cycle D, H, a, A, B, C, D. 3. Endogenous budding: buds retained within shell, cycle D, J, a, A, B, C, D. 4. Bud formation outside the shell, cycle D. J, a, A, B, C, D. These are all vegetative or schizogony methods of reproduction and occur in summer months. 5. Conjugation of buds, cycle D, K, L, a, A, B, C, D. 6. Chromidiogamy or mixing of chromatin: cycle D, M, N, O, P, Q, a, A, B, C, D. Terminology: microgamete is the male uniting cell, macrogamete is the female uniting cell. Product of sexual union is a zygote or ookinete: i.e. a protozoal egg. Arcella simple example of diversity of modes of reproduction.

Difflugia another similar example of diversity of life cycles. 1. Binary fission. 2. Contents of one Difflugia flow into second, leave behind empty shell. Nuclei break down into idiochromidia. Idiochromidia of both individuals fuse together to form new nucleus of zygote. Thick cyst wall secreted. Rests till spring: divides then inside cyst into 2, 4, 8, 16, 32 etc. daughter amœbulæ, which creep out and grow into adult Difflugias.

Association of protozoal individuals. Three clearly defined and different types. 1. Mingling of cytoplasms (only), termed plasmogamy or plastogamy. 2. Interchange of cytoplasm and of nuclear material between two cells, as in the conjugation of the Ciliophora. 3. True sexual conjugation or syngamy: the criterion for which is fusion of male and female chromatin from two different individuals to form a new, single individual, or zygote. Isogamy if the uniting male and female gametes are alike and cannot be differentiated (as in the Gregarines): anisogamy if, as is more usual, they are different in size and appearance.

Free living amœbæ, especially of the "limax type" may rarely be found in human fæces. They have nothing whatever to do with the patient. They may come from dirty water used to wash the bed pan (vide Dobell on amœbiasis of the bed pan and urinal); or their tough walled cysts may be ingested in dirty water or vegetables. These cysts then pass unaltered through the intestine: pass out in the fæces and there in favourable conditions of air and moisture de-excyst and free amœbæ creep out. The finding of such free living amœbæ in stools is rare: but one must be prepared for them at any time. Dobell terms them "coprozoic": i.e., living, not in the intestine, but on the passed faeces. Dobell describes three as having been reported from passed human stools: viz:—

1. Dimastigamœba gruberi. Small amœba, 7 to 15 μ, nucleus with big central karyosome, amœba with big contractile vacuole. Pseudopodia large and consist of only ectoplasm: endoplasm stuffed with food particles and bacteria. Multiplies by binary fission. If environment gets very fluid this amœba peculiar in that it assumes an ovoid shape, produces two flagella and moves by flagellate as well as by amœboid movement. Cyst is 8 to 12 μ: outer thick rough wall, inner smooth wall, wall is perforated by 3 to 8 little pores. Inside is one encysted amœba with big vesicular nucleus.

2. Hartmannella hyalina. 9 to 17 μ in size, single contractile vacuole, vesicular nucleus with big central karyosome. Multiplies in vegetative state by binary fission. Cyst 10 to 14 μ: outer wall very thick and wrinkled, inner wall smooth and thin. Inside single amœba with big vesicular nucleus and some deeply staining dots=plastids.

3. Sappinia dipolidea. Larger. 10 to 30 μ. Much ectoplasm which is very dense and solid and hence surface knobbed and wrinkled. Endoplasm full of food particles. Always possesses TWO nuclei, each similar: vesicular with big karyosome. Multiplies by binary fission into two: each daughter amœba has two nuclei. Cyst is peculiar. Contains two individuals inside same cyst: each with two nuclei. When cyst ruptures however only one set free, with two nuclei ?? does conjugation occur within the cyst. (This amœba is to be found in Calcutta in the tank near the University buildings).

A fourth free living, coprozoic amœba, is common is fæces of toads and frogs, and occurs in India, Chlamydophrys stercorea. Is one of the shelled amœbæ: i.e. secretes a thick porcelain-like shell: (N.B.—this is not a cell wall: it is not living, but is a secreted exo-skeleton). At times and with young forms is not shelled. Pseudopodia protrude through opening at narrow pole : are ray-like. Has one big vesicular nucleus with big karyosome.

These coprozoic amœbæ are quite unimportant : but they may at times be found in stools : are characterised by vegetative form having a contractile vacuole, and cysts having thick, rugose walls : nothing like the true entamœbæ of man. Most of the free living amœbæ can be readily cultivated : Musgrave and Clegg's medium=Agar, 2.5 gms., salt 0.05 gm., Lemco 0.05 gm., normal NaOH solution 2 c.c., water to 100 c.c. Autoclave and pour into Petri dishes. Inoculate from e.g. decaying cabbage matter. Invert over the culture the upper part of Petri dish in which place moistened filter paper. Or simple 5 % egg albumin a good medium. The free living amœbæ will grow in almost any hay or vegetable infusion. Workers in past years have very frequently inoculated such media from human stool : got them accidentally contaminated with free living amœbæ : mistakenly describe such as human entamœbæ. Are nothing of the sort : mere accidents. Free living amœbæ have :—1. contractile vacuole, 2. big excentric vesicular nucleus, often near centre of amœba, 3. cysts with rugged, tough cyst walls.

Water and food supplies being full of amœbæ in course of eons of evolution certain amœbæ became inhabitants of the intestinal tracts of man, animals, reptiles, etc. By degrees these amœbæ became SPECIALISED PARASITES EACH FOR ITS OWN HOST AND FOR THAT HOST ALONE. These constitute the ENTAMŒBÆ : i.e. amœbæ which live only in a given host. Further the entamœbæ are each SPECIFIC FOR ITS OWN HOST. Thus E. ranarum of the frog very much resembles E. histolytica of man : but Dobell has shewn that E. ranarum will not parasitise man : nor will E. histolytica of man parasitise the frog. The entamœbæ exist outside their own hosts ONLY IN THE FORM OF RESISTANT CYSTS AWAITING BEING SWALLOWED BY THEIR OWN HOST. Recent amusing incident of a jail superintendent who thought outbreak of dysentery was amœbic : said he hasd found motile E. histolytica in the jail water supply. Only form of E. histolytica which he could have thus found would have been the cyst : the motile form dies at once on leaving body : had mistaken a free living natural amœba of the water supply for E. histolytica. Whole animal world full of instances of parasitic entamœbæ. E. blattæ, with much clear ectoplasm, and big pseudopodia found in fresh dissections of rectum of majority, of all cockroaches. E. muris of rat. E. ranarum of frog very common : and is in many ways morphologically like E. histolytica of man. Note, in passing that character of pseudopodia (upon which so many authors rely) is apt to vary with environment : thus E. blattæ has big blunt pseudopodia : but if distilled water added pseudopodia become thin and finger like. E. blattæ very convenient parasite for study. Lives in motile, vegetative stage in lumen of cockroach hind gut : multiplies by binary fission : produces cyst which is passed in fæces : cyst in outside environment withstands partial desiccation : swallowed by fresh cockroach : excysts : second cockroach infected.

Before we begin study of parasitic protozoa note, 1. free living amœbæ : on very rare occasions seen in human stools, i.e. coprozoic amœbæ, nothing to do with man in reality at all, 2. protozoa may be epizoic, i.e. living on the surface of the body of an animal but entirely harmless, 3. they may be entozoic, living in the parasitised animal.

But even when entozoic, they are not necessarily harmful. Nor are they necessarily really parasitic. Of the five entamœbæ which live in the human gut only one causes disease : and even then only exceptionally. Entamœba histolytica lives in mucous wall of colon of some 10% of humanity : in 90% of whom it causes no symptoms : host and parasite usually mutually adapt themselves : i.e. state of commensalism. E. coli, for instance, eats bacteria, yeasts, never causes disease : may be actually beneficial to its human host. (If so then why, in the name of everything diagnose amœbic dysentery because E. coli is found in the stools and go in for drastic courses of emetine which depress the patient and do not affect the entamœba ??). Even with such virulent parasites as E. histolytica which are capable of producing fatal dysentery, the LAST THING WHICH THE AMŒBA DESIRES IS ACUTE AMŒBIC DYSENTERY IN THE PATIENT FOLLOWED BY DEATH. If the patient dies, the E. histolytica also dies. All Nature's arrangements directed more to a mutual balance—commensalism—than to disease : disease, the result of disturbance of harmony in relationship between parasite and host.

APPALLING MUDDLE OF LITERATURE ON ENTAMŒBÆ OF MAN. E. histolytica described under 34 different names, E. coli under 23. Cunningham, 1871, in stools of cholera patients, Calcutta, discovered E. coli : called it "Amœba coli." Lœsch in Petrograd in 1875, discovered E. histolytica : called IT ALSO "Amœba coli." This the beginning of the muddle. Worker after worker, working with stale stools, dying entamœbæ and old material described supposedly new species after species. Thus: E. tetragena, Viereck, 1908= degenerating E. histolytica. E. minuta, Elmassian, 1909= pre-cystic stage of E. histolytica, E. tenuis, Kuenen and Swellengrebel, 1917=E. histolytica. Chatterji's "new amœba which causes liver abscess"=E. histolytica in binary fission shewing two nuclei: Kofoid's new Councilmania lafleuri= E. coli. From Cunningham's original discovery, 1871, to 1900 whole literature much more accurate than literature from 1900-1914. Castellani's E. undulans=degenerating phases of Trichomonas hominis (flagellate). Knowles and Cole, owing to APPALLING muddle of literature, and with no clear description of E. coli and E. histolytica to guide them, in investigating convalescent dysentery cases in Bombay, end of 1916 concluded E. coli=E. histolytica=E. "coli communis." Were wrong. Then came war papers of Dobell, (10,000 stools of convalescent dysentery cases examined in four years): Wenyon and O'Connor at Alexandria. Result was to clear up whole incredible confusion. Then came Dobell's books of 1917 and 1921. May now be taken as standard and unquestionably leading authority. (Much of confusion due to Schaudinn's celebrated 1903 paper on human entamœbæ: he described and used names E. coli and E. histolytica: but gave accounts of life cycle of E. histolytica which don't exist—are only degenerations: and described parts of E. histolytica life cycle for E. coli: commenced the muddle). Advise students to discard all literature prior to 1917: and take Dobell as standard, authoritative writer.

If so there are six entamœbæ of man: (and six only: there may possibly be more: but none of them has been proved). THEY ARE SPECIFIC TO MAN AND EXIST NOWHERE ELSE,—except in outside world as cysts. One—E. gingivalis—inhabits the mouth: the other five, Endolimax nana, Iodamœba butschlii, Dientamœba fragilis, Entamœba coli and Entamœba hystolytica inhabit the human (and no other) intestine. Classify:—1. Order Amœbæ. Sub-Order Entamœbæ. Genus Endolimax. Nucleus with big, irregular, excentric karyosome, Only (human) species, E. nana. 2. Order Amœbæ. Sub-Order Entamœbæ. Genus Iodamœba. Nucleus with a big, central karyosome, surrounded by chromatin granules. Only (human) species, I. butschlii. 3. Order Amœbæ. Sub-Order Entamœbæ. Genus Dientamœba. Entamœbæ which have two nuclei (instead of one) in the vegetative state. Only (human) species, D. fragilis. 4. Order Amœbæ. Sub-Order Entamœbæ. Genus Entamœba. Entamœbæ with "ring" type of nucleus (i.e. with small spherical karyosome). Only (human) species :—E. coli, E. histolytica, E. gingivalis.

Will take them up in this order, reserving comparison bebetween E. coli and E. histolytica to last.

Note here : in measuring under the microscope size of minute objects we use the Greek letter "μ." Method of measuring will be shewn in demonstration specimens. One μ=one thousandth part of one millimetre. Average diameter of an R. B. C.=7 to 8 μ as standard for easy comparison.

1. *Endolimax nana.* Wenyon and O'Connor, 1917. Lives in the SMALL intestine, in the lumen of the gut. Vegetative form :—size 6 to 12 μ. Sluggish motility. Ectoplasm scarcely seen : if seen (in diarrhœic stools in fresh state) shews blunt, tiny pseudopodia of ectoplasm only. ABOUT THE SIZE OF AN R. B. C. IN DIAMETER. Greenish colour : never ingests R. B. Cs. : endoplasm stufiffed with food particles. (N.B.—Often parasitised by a minute Sphaerita when we get appearance of mulberry-like mass, staining black with Iron hæmatoxylin in the endoplasm : have seen this in some Calcutta specimens). Ingests bacteria, yeasts, etc. Vacuoles usually well seen. Nucleus consists of a blob-like mass of chromatin =karyosome, excentrically placed towards the nuclear membrane Nuclei of different individuals vary from one to another. In fresh, motile state nucleus seen as dull blob=karyosome. Probably multiplies in motile state whilst in small intestine by binary fission : but process has still to be described. Never attacks tissues and is quite harmless. Very common entamœba : some 25 to 30% of healthy persons are infected with this entamœba.

As motile entamœbæ come down into colon they commence to encyst. Encystment phase of all entamœbæ=their minuta phase. All foreign food particles cleared from ectoplasm, which becomes glassy clear, vaculoes got rid of, amœba rounds up and becomes motionless little spherical ball. Later becomes elliptical or oval. At first cyst is mononucleate. Secretes thin, scarcely visible cyst wall. Ceases to move. By degrees nucleus divideds into two, then four. (Glycogen vacuole and chromatoid bodies : see later : are rare in E. nana cysts).

E. nana typical cyst : seen in formed stools. Usually oval, sometimes spherical, about 8 to 10 μ long, 7 to 8 μ wide. No glycogen. Chromatoid bodies, if any, very rare, filamentous. Has four nuclei and thin cyst wall : nuclei often gathered together towards one pole of cyst. (Very, very exceptionally, nuclear division may overshoot the mark and go on to eight nucleate cyst). As seen in fresh stool is oval, about size of R. B. C., nuclei barely visible.

Cyst often contains a few brightly refractile volutin granules =reserve food particles.

Complete desiccation kills the cysts : but nothing short of it. Cyst passed in formed stools : lies in dust etc., swallowed by new host, who gets infected. Cyst ruptures in small intestine, liberting amœboid amœba. ? Possibly four small amœbulæ emerge : no abservations made.

Wenyon found E. nana in 33% convalenscent intestinal cases, British troops, in Alexandria during the war. No evidence that it has any association with disease. Remember its small size, about that of an R. B. C., sluggish motility, and tetranucleate, usually oval cyst.

Practical Notes.

In examininig stools for protozoa :—

1. NEVER omit to examime fresh saline emulsion. Stool should be absolutely fresh, and FREE FROM URINE, which tends to kill off motile amœbæ. Take a perfectly clean slide : on it put small drop of saline : with end of scalpel or needle make fine emulsion of suspicious particle of stool. If stool dysenteric examine blood stained mucus. Make a THIN emulsion, (it ought to be easy to read print through it). Cover with perfectly clean cover slip : examine with condenser down, concave mirror, work systematically from one corner of cover slip to other. If the stool is fluid or very mcuoid simply mount a drop under cover slip without saline.

2. Make a similar emulsion in Iodine 1 gm. : Potass. Iodide 2 gms., water 100 c.c. solution. Nuclei of cysts etc. stain, flagella shew up. Iodine preparations are expecially useful for identifying nature of cysts seen in saline preparations. Or :—make a fine emulsion of stool in pestle and mortar in iodine solution : emulsify and then centrifuge thoroughly : pick up tiny particle of deposit : emulsify in saline under cover slip : protozoa, iodine stained, stand out against colourless background.

3. Three or four preparations from all suspicious parts of the stool should be examined. Cysts are seen in formed stools, motile protozoa in dysenteric and diarrhœic ones.

Literature.

Only two reliable accounts of the human entamœbae exist: viz :—

Dobell, C., 1919. The Amœbae Living in Man. John Bale, Sons & Danielsson.

Dobell, C., & O'Conner, F. W., 1921. The Intestinal Protozoa of man. John Bale, Sons & Danielsson.

Both books contain a very complete bibliography.

LECTURE 3.

IODAMŒBA BUTSCHLII, DIENTAMŒBA FRAGILIS, ENTAMŒBA COLI, ENTAMŒBA HISTOLYTICA.

2. *Iodambœa butschlii.* Discovery of "Iodine cysts" in stools by Wenyon and O'Connor, 1915. Dobell, 1917, shewed that Iodine cyst=cyst of entamœba, I. butshclii. Wenyon found it in 3% healthy troops, Alexandria, 15% jail prisoners, 7% regimental cooks, 5% convalescent soldiers. No evidence to connect it in any way with disease=harmless commensal. Vegetative form (only seen in unformed stools). 9 to 15 μ, sluggishly motile, ectoplasm well seen in moving amœba, but, as amœba soon dies after leaving body not often seen. Pseudopodia blunt=ectoplasm only. Colour very refractile. Endoplasm stuffed with bacteria, food particles etc. (never R. B. Cs.) and always very vacuolated. Nucleus difficult to make out in fresh specimen, When stained is large, with much chromatin. Almost all the chromatin=large central nucleolus or karyosome : around this clear zone with few chromatin dots : then around all well defined nuclear membrane. In many ways the nucleus of I. butshlii recalls that of a free living amœba. In vegetative state probably multiplies by binary fission in colon, but process not yet described.

As it comes down gut it encysts=characteristic Iodine cyst. All food particles thrown out, rounds up to some extent, (but often of erratic shapes), does *not* get smaller. Fresh specimens are very clear with glassy protoplasm and, if stained, shew very big nucleus. Next thick cyst wall secreted, bright refractile volutin granules appear in cytoplasm, and glycogenic iodine vacuole forms.

Cysts very characteristic structures. Thick wall, irregular shapes, and often met with in clusters as if they came from the same patch of colon wall. One nucleus only,=differential point from E. coli cyst at 2 nucleate stage, which sometimes resembles it. 9 to 12 μ. Lying in the cytoplasm is a vacuole of varying size, usually very big, very rarely indeed absent, filled with glycogen. Sometimes two glycogen vacuoles.

Glycogen is reserve food stuff for cyst. Dissolves in water: hence in specimens stained with watery stains only seen as big, empty vacuole. Proved to be glycogen by being soluble in water, insoluble in alcohol (hence if Iodine cyst suspected emulsify in Tinct. Iodii and examine), stains rose red with Best's carmine stain. In addition to big glycogen vacuole or iodophilic body cyst shews bright, refractile volutin granules, and, in cytoplasm to one side of glycogen vacuole is the single nucleus. Nucleus is almost invisible in fresh state, but when stained shews chromatin massed together as big lump, applied to one side on inner aspect of nuclear membrane. The nucleus of the cyst differs from that in the vegetative form. The appearance is that of a signet ring with a large seal.

In saline emulsion living cyst shews irregular contour, thick cyst wall, dull area of glycogen vacuole, bright volutin beads. In iodine emulsion cyst shews up very strikingly. (Might be mistaken for 2 nucleate E. coli cyst which also has big glycogen vacuole: but different nucleus and I. butschlii cyst has only one nucleus).

I. Butshlii found in 2½% Calcutta cases. It has one striking peculiarity: a single injection of emetine eradicates the infection from the patient.

In iodine I. butschili cysts have a peculiar greenish tinge: whilst the glycogen vacuole stains a deep mahogany brown.

3. *Dientamoeba fragilis*, Dobell and Jepps, 1918. Again harmless commensal. So far only discovered in some 8 cases Name describes it. Vegetative amœba 8 to 9 μ: very actively motile when fresh, very clear, very flimsy, voluminous ectoplasm. Pseudopodia fragile, broad, leaf like. Dies at once when passed in stool. If seen perfectly fresh travels rapidly with rounded granular endoplasm to one side, and broad fragile pseduopodia streaming out, like a snail in movement. Endoplasm granular and flimsy also, full of bacteria and food particles. As it dies as soon as passed in stool soon assumes appearance of big bladder-like degenerated vesicle. Has TWO nuclei: whence Dientamœba as genus, and fragilis from fragile character. Both nuclei similar: each has well marked nuclear membrane, and big, star-like central cluster of chromatin. Nuclei well seen as bright referactile blobs in fresh specimen. Very rarely forms met with only one nucleus. Process of multiplication and cyst quite unknown. As it dies in passed stool cytoplasm becomes bubbly, intensely vacuolated, and whole organism becomes merely a big vacuole with rim of cytoplasm around.

Only importance of D. fragilis is that one may suddenly discover it any day in India.

Above entamœbæ belong to three different genera. Dobell confines the name Entamœba (proper) for a genus of entamœbæ characterised by having a ring-like type of nucleus with a small spherical karyosome.

4. *Entamœba coli.* Incidence in some 20 to 25% of all humanity. Formerly regarded as commonest protozoon of man, but quite possibly E. nana is commoner. Clacutta figures (1922) gave 12% incidence. Lives in lumen of colon and is perfectly harmless commensal, indeed possibly useful parasite as intestinal scavenger. (Some authorities believe, but doubtful, that E. coli " infections " can be " cured " by heroic treatment with emetine for weeks orally plus hypodermically, but " treatment " far worse than perfectly harmless infestation).

Discovered by Lewis, 1870, in dejecta of cholera cases, Calcutta. Wenyon's incidence figures are :—20% healthy British troops, 39% convalescent intestinal cases, 49% jail prisoners. (Almost every Indian sweeper will provide material for its study).

Schaudinn's 1903 paper laid whole basis of muddle of literature on entamœbæ and misled all subsequent workers. True life history of E. coli and correct morphology worked out by James, Darling, Wenyon and O'Connor 1915-19 : and Dobell's account the only reliable one.

E. coli lives in lumen of upper part of colon, where gut contents are fluid. In motile state only to be seen in soft or fluid stools. Largest entamœba of man, 20 to 40 μ ; (N.B. Any entamœba smaller than 10 μ is not E. coli). Sluggishly motile. Only a mere trace of ectoplasm. Pseudopodia are big, rounded, dome shaped and shew very little ectoplasm, and consist of endoplasm chiefly (contrast E. histolytica later). Appearance in fresh state greenish, ground glass-like. Endoplasm densely granular and packed cram full with all sorts of food stuffs, bacteria, yeasts, debris, but never R. B. Cs. Voracious feeder. Endoplasm full of vacuoles, mostly food vacuoles, and these are often cleft or crack-like Nucleus very prominent : in fresh unstained specimen shews up clearly as bright, circular or slightly oval, refractile ring=chromatin deposit on nuclear membrane. Stained " coli type " of nucleus =big spherical karyosome situated excentrically, and NOT in centre of nucleus. Around it is clear halo. Between this and nuclear membrane come fine chromatin grains. On inner surface of nuclear membrane is thick, uniform (but in badly fixed specimens often bead-like) deposit of chromatin = bright refractile ring seen in unstained state.

Multiplies probably by binary fission, but complete process not yet described. So voracious a feeder is E. coli that intestinal flagellates, cysts of Giardia, Chilomastix, Isospora and even cysts of E. histolytica have been described as phagocytosed by it.

As E. coli comes down the gut into solid fæces it prepares to encyst. Pre-cystic stage. Pseudopodia withdrawn, all food particles and debris cleared, vacuoles disappear, endoplasm becomes very clear and very refractile. Amœba becomes motionless and rounds up into little ball = minuta phase. AT THIS STAGE IT IS EXCEEDINGLY DIFFICULT TO DIFFERENTIATE THE PRE-CYST OF E. COLI FROM THE PRE-CYST OF E. HISTOLYTICA, and it is just THIS FACT which is the basis of the whole confusion in the literature. The clearly visible nucleus of E. coli and its nuclear pattern on staining are the two best guides to distinguish it. The precyst of E. coli is 12 to 17 μ with single, rather big, easily visible nucleus.

Next nucleus divides into two = binucleate stage, and cyst wall is secreted. Cyst is larger than pre-cyst. As this happens a more or less big glycogen vacuole forms, and TYPICALLY AT THE BINUCLEATE PHASE WE HAVE A SPHERICAL CYST WITH TWO NUCLEI AT OPPOSITE POLES SEPARATED BY A BIG PROMINENT GLYCOGEN VACUOLE. Soft stools from E. coli cases may often be found swarming with E. coli cysts at this stage. Sometimes there is no glycogen. There may also be a big glycogen vacuole at the mononucleate stage.

Next 2 nuclei divide into 4 nuclei. The 4 nucleate stage is very rapidly passed through and is the rarest form of E. coli cyst seen in the stool. At this stage the glycogen is very rapidly used up and only traces of it are seen, whilst at the 8 nucleate phase it has gone.

Next nucleus divides again and we get typical CHARACTERISTIC 8 NUCLEATE ADULT E. COLI CYST. E. coli infection diagnosed in 98% of cases by finding typical 8 nucleate cyst in stools. On rare occasions nuclear division over hoots the mark and E. coli goes on to produce giant, and often ovoid or elliptical cysts with 16 or even more nuclei.

Typical adult 8 nucleate cyst is round (rarely a little oval), 15 to 22 μ (there are three races in size with 16, 19 and 22 μ diameters), 8 nuclei, very prominent in the fresh specimen and when stained, with reproduction of typical E. coli type of nucleus, no glycogen or only the merest trace of it. CHROMATOID BODIES ARE VERY RARE IN E. COLI CYSTS. They do occur in some 5% of E. coli cysts and are then of a fine, feathery form, like splintered glass or bundle of faggots. They stain black with Iron hæmatoxylin and again = food reserve.

As seen in iodine the adult E. coli cyst has a peculiar finely granular appearance that is quite unlike the smoother and more hyaline appearance of the E. histolytica cyst.

87% of E coli cysts seen in stools are at the 8 nucleate stage. Finding will depend on intestinal state of patient. In diarrhœic stool we may get motile, vegetative stage. In soft stool very often whole stool filled with 2 nucleate cyst stage. 4 nucleate stage rarely seen. Formed stool full of 8 nucleate cysts. Cyst very easily distinguished and unmistakeable.

Importance of realising true life cycle of the human entamœbæ. When stool is passed all vegetative E. coli die : also all pre-cysts, 1, 2 and 4 nucleate non-adult cysts = all are non viable. Of the adult 8 nucleate cysts many die but perhaps half survive. Complete desiccation will kill cyst but nothing less : it survives at least a month in water or damp.

Classical experiments of Walker and Sellards in Philippines, 1913. Took 20 jail prisoners and made certain by repeated examination of their stools that they had no intestinal protozoa of their own. Fed on fully cooked food, drank only distilled water, to exclude all outside sources of infection. Then given capsules containing fresh fæces full of E. coli cysts. 17 out of the 20 become parasitised by E. coli, and commenced to pass E. coli in stools in from 4 to 11 days time :—average incubation period 4 to 7 days.

Much other and often very inaccurate work on E. coli. Yoshida 1918 and 1920, washed cysts with 2% HCl and placed in special medium in running stream and describes process of excystation : but his plates are those of degeneration and not life. Schaudinn described autogamy, fusion of nuclei together within the cyst ; but was almost certainly dealing with dying cysts. Kofoid describes mode of division of cyst nuclei by karyokinesis and says there are definite chromosomes but ?? Mathis and Mercer describe male and female cysts but again ???

Kuenen and Swellengrebel's test for viable cysts : emulsify in 1% watery eosin. Dead cysts stain, living ones don't, Reliability a little doubtful.

E. coli absolutely limited to man. A rather similar entamœba has been described from apes, but is almost certainly one peculiar to apes and nothing to do with E. coli. All animal experiments negative. Two main bits of research work wanted with reference to E. coli :—1. to get fresh material from upper colon in operating theatre and study and describe process of multiplication in vegetative stage : and 2 to ascertain exact process of de-excystation. The cysts pass unaltered through the stomach into small intestine Here the cyst wall gives way. What emerges ? Do 8 little new E. coli emerge, or do some of the nuclei fuse Yoshida says only one amœba emerges : 6 nuclei go to bits, other two fuse, mononucleate amœba emerges, but his plates don't carry any conviction.

5 *Entamœba histolytica* One of the most deadly parasites of man, cause of amœbic dysentery, amœbic abscess of liver, spleen (Rogers and K. K. Chatterji), brain, and amœbiasis. Called histolytica because of its remarkable tissue-dissolving powers. Discovered by Lœsch 1875, in fatal case of amœbic dysentery in Petrograd. Unfortunately he called it Amœba coli. Literature prior to 1915 on this amœba, and much of the later stuff also is simply confusion worse confounded. THE REAL REASON WHY THE LITERATURE ON THE ENTAMŒBÆ IS SO BAD IS THAT WORKERS DO NOT, WORK WITH FRESH STOOLS. All the entamœbæ (with the exception of their adult cysts) die very soon after leaving the colon : workers get hold of these dying amœbæ, fix and stain them very imperfectly and then describe wonderful new species and even more curious life cycles which don't exist, but are simply pictures of cell degeneration and death. The REAL WAY TO STUDY E. HISTOLYTICA IF YOU CAN'T GET ABSOLUYTELY FRESH MATERIAL IS TO INFECT A KITTEN, when one gets the real living entamœba in its pristine state.

E. histolytica lives in man IN THE MUCOUS MEMBRANE OF THE COLON. (N.B.—Entirely different from other entamœbæ which live in lumen of the gut). Occurs in the human colon :—1. in association with and as cause of acute amœbic dysentery, when actively motile E. histolytica in vegetative stage are passed in the stools : 2. in convalescence after amœbic dysentery, when the usual finding in the stools is pre-cysts : 3. in the convalescent carrier, when the adult 4 nucleate cysts are found in the stools : (N.B. THE CYSTS IN THE CARRIER'S STOOLS BEING PRODUCED FROM

ACTIVE VEGETATIVE MOTILE E. HISTOLYTICA LIVING IN HIS COLON MUCOSA 4. in apparently healthy persons who have been in contact with amœbic cases=contact carrier, cysts in stool : and 5 in perfectly healthy persons who have never been near amœbic cases, e.g. in England, where again cysts in stools. Only some 10% of persons infected with E. histolytica ever shew any symptoms of amœbiasis. In 90% of persons infected with this parasite it does not cause sufficient tissue destruction to cause symptoms=carrier state. Obviously death of the patient=death of his histolytica and is the last thing to suit the parasite, which would much rather live in a state of balanced parasitism on the patient.

E. histolytica infection now known to be world wide: United Kingdom, Russia, France, Germany, U. S. A., even Greenland and found among the Esquimaux as well as throughout tropics and sub-tropics. Some 10% of humanity are parasitised by E. histolytica. Calcutta 1922 figures, are 24 out of 233 cases examined. Wenyon's figures are 5% healthy British troops, 10% regimental cooks (most dangerous), 14% of apparently healthy jail prisoners. Carrier state has been shewn to persist for, in 3 cases, 18 months, 5 years, 16 years and in one case probably for 34 years respectively, and it is an open question as to whether in an untreated carrier the condition may not persist for life.

Importance of realising what is meant by a histolytica carrier. The active, motile, vegetative E. histolytica are living in the mucous membrane of his colon. They are destroying it. But at the same time Nature is repairing it. Balanced condition of tissue destruction plus tissue repair amid which the E. histolytica lives, patient " like a modern Prometheus " to quote Colonel Alcock. From these active entamœbæ in the gut cysts are produced which are passed in the stools. Term " cyst carrier " so much used in the literature has no meaning at all, and entirely wrong: cf. hookworm infection; we don't talk of a hookworm infected person as an egg carrier. Proper term is E. histolytica carrier. Yet read general medical literature and you will see that the misuse of terms has led medical men generally to think that the carrier is carrying cysts.

The carrier is a danger both to himself and others. At any moment, from causes as yet not understood, (but almost certainly dependent on the pH of the gut wall), conditions may become extra favourable for the parasite and carrier goes down with amœbic dysentery or hepatic abscess. He is intermittently passing E. histolytica cysts in his stools, and these cysts are infective to other persons. Wenyon and others emphasise importance of flies, passing from fæces to food; carrying histolytica cysts on their feet. But in India always where you get a patient suffering repeatedly from amœbic dysentery you will find that someone who has the handling of his food is a histolytica carrier. Vegetative amœba, then, lives in and on the mucosa of the colon. Size very variable, 20 to 30 μ, sometimes less than 10 μ, smaller than E. coli, but size little use to distinguish. It is exceedingly motile: if seen in perfectly fresh stool from infected kitten it moves across field with great rapidity, throwing out fine, long, finger or ribbon-like pseudopodia. One third of parasite consists of clear, glassy, voluminous ectoplasm, a most useful point in diagnosis. When seen in sections of the infected gut wall however there is little distinction between ecto- and endoplasm. It is only as seen in the passed stool that the ectoplasm shews up so clearly. Colour clear, glassy, often yellowish from included HB. Endoplasm finely granular with few, scanty, spherical vacuoles. Feeds :—1. it secretes a powerful proteolytic ferment which dissolves the mucosal epithelial cells. In sections of infected gut the entamœbæ are seen to lie in little pools of dissolved tissue : feeding by osmosis on the richly nutritive fluid thus produced. Acton draws attention to "pre-adult", small vegetative forms with vacuoles full of prezymogen : 2. it also bodily ingests R. B. Cs., which come to lie in food vacuoles in its endoplasm and are gradually dissolved up. E. histolytica is a dainty, selective feeder. E. histolytica has no other articles of diet, ANY ENTAMŒBA CONTAINING INCLUDED R. B. Cs. IS IPSO FACTO E. HISTOLYTICA, (N.B. Entamœba, not Amœba). In its fresh living state E. HISTOLYTICA NEVER INGESTS BACTERIA, YEASTS, FUNGI OR SIMILAR FOOD PARTICLES. ITS PROTOPLASM IS PERFECTLY STERILE. This is perhaps the most important point about the parasite : (hence pus of amœbic liver abscess is always sterile, unless secondary contamination has occurred).

The nucleus is invisible in the fresh state. When stained it is of "histolytica type :" central, very fine, tiny karyosome : around it pale clear halo, around this clear zone to nuclear membrane. On inner surface of nuclear membrane is a very thin, fine, uniform deposit of chromatin. Contrast "coli type." "Histolytica" nucleus is much more delicate pattern : indeed at every point histolytica is much more delicate and finer and more specialised parasite than coli.

Multiples in tissues by binary fission. Process partially seen once only in 15 years of study by Wenyon in a fresh stool: but finally discovered and worked out by Dobell in sections of gut wall of infected kitten. Also once seen in kitten stool (Knowles 1923): whilst G. C. Chatterji saw it in liver abscess wall sections. Nuclear division gives a picture resembling mitosis. Almost true karyokinesis. Kofoid describes chromosomes but ?

Above is the TRUE picture of vegetative, motile, active E. histolytica. Only writer who describes it is Dobell. Suppose this motile, living entamœba now gets out of gut wall and is passed in the stool. IT AT ONCE COMMENCES TO DIE. In about 3 hours or less it is dead. Motile E. histolytica as studied in any stool passed more than 30 minutes previously is dying E. histolytica, and NOW PRESENTS AN ENTIRELY DIFFERENT PICTURE. It becomes sluggishly motile. Pseudopodia become big, massive, dome-like, voluminous: but ALWAYS CONSIST ONLY OF ECTOPLASM, a most important point in identification. The endoplasm becomes much vacuolated, vacuoles running together and coalescing but still spherical. Bacteria, fungi, yeasts in the stool now commence to INVADE THE DYING ENTAMŒBA. Hence motile, dying E. histolytica as seen in stools often shews bacteria, yeasts etc., but this is NOT phagocytosis by the entamœba, it is phagocytosis of the entamœba by the bacteria etc. By degrees the endoplasm gets more and more vacuolated. The nucleus, hitherto invisible in fresh state, becomes visible as a thin, delicate ring, becomes degenerated, goes to pieces. Final stage (as seen in stool say 6 hours old) is a rounded up, immobile bit of vacuolated protoplasm, consisting of almost all ectoplasm and stuffed with invading bacteria, fungi etc. It is this dying E. histolytica which Hartmann, 1908, mistook for a new species of entamœba, (described as having characters exactly midway between E. coli and E. histolytica). In reality E. tetragena=E. histolytica=E. minuta.

References.

Yoshida's two papers will be found in the Jl. Expl. Medicine, 1918, Vol. 28. p. 387. and Archives Internal Medicine. 1920. Vol. 32. p. 357.

Practical Notes.

Examination of stools for Intestinal Protozoa. Rule 1. Always insist on getting fresh stools for examination. 2. There should be no urine in the bed pan. 3. If possible get the whole stool. Cysts will be found in formed stools, (examine piece from centre) : motile protozoa in soft and fluid stools. 4. Never omit to record macroscopic character of stool. 5. Often useful to test the stool with litmus paper, amœbic dysentery stools usually acid to litmus, bacillary dysentery stools usually alkaline.

1. Take suspicious portions of stool, e.g. mucus, blood stained part, etc. On perfectly clean slide make a very THIN emulsion in a drop of saline : (if mucus or if very fluid stool, addition of saline not needed) : cover with cover slip, and examine systematically on mechanical stage. The emulsion must be so thin that print can easily be read through it, and cover slip, lenses etc. must be clean. A thick emulsion, dirty cover slip, and dirty lenses will spoil all work. Motile protozoa seen in this emulsion. (Concave mirror, condenser racked down, iris perhaps half closed). If cover slip thin and preparation good it will stand examination with the oil immersion lens. The addition of 1 in about 600 fluorescein to the saline sometimes useful : fluorescein stains all dead cells, intestinal epithelium, leucocytes etc. (cf. its use to detect corneal ulcer), but does not stain living protozoa. NEVER OMIT THE EXAMINATION IN FRESH SALINE.

2. Make a second similar very thin emulsion in solution :—Iodine 1, Potass. iodide 2, water 100 parts. Or—better still make thin emulsion of one part stool in ten parts of iodine solution : centrifuge : throw away supernatant fluid, mount small particle of iodine stained deposit in saline under cover slip. This gives iodine stained protozoa against colourless background. The iodine stains up nuclei, e.g. the nucleus of E. histolytica ; shews up flagella of flagellate protozoa and greatly assists diagnosis. Use the iodine strong enough : weak solution fails, and solution decolourises in light. **RECOMMEND TO ALWAYS FIRST EXAMINE IN SALINE, THEN IN IODINE, AND DO BOTH FOR EVERY STOOL.** Motile protozoa well seen in 1, encysted protozoa in 2.

3. Everything depends on 1. perfectly clean slides, lenses etc., 2. a really thin emulsion, and 3. getting critical illumination. Given this one can use the oil immerson lens, after the $\frac{1}{6}$th.

4. Stitt recommends combining freshly mixed saturated aqueous eosin with equal part 5% potass. iodide solution saturated with iodine and emulsifying in this. Protozoa stain brown against pink background.

(Read and study Chapter 7 of Dobell's Intestinal Protozoa).

5. Take a perfectly clean slide. On it place tiny drop of either egg albumin fixative, or—better—fresh serum. With clean finger smear as very thin film over slide. Over this spread thin emulsion of the stool, as smear. If motile protozoa are present, turn film upside down and expose two minutes to Osmo-chromic acid vapour to kill in situ. Let the film now dry. Fix by dropping on to it methyl alcohol : leave on for a minute : wash off with water : soak the film in distilled water. Is now ready for staining, as follows :—Either :—

A. Use Leishman's stain. Pour stain on to film to cover evenly. Count 15 slowly. Add equal part distilled water and mix. Allow to stain ten minutes. Wash out very thoroughly in distilled water. If it is found that the film is now very blue it can often be improved by treating with 1—1,000 acetic acid and then thoroughly wash under tap.

B. Giemsa's stain. Add one drop to each c.c. distilled water. Flood slide. Stain 20 to 30 minutes or more. Wash out and differentiate as above in distilled water.

Leishman's and Giemsa's stains are hit and miss with fæcal films. E. coli and E. histolytica stain very badly with them. E. nana and I. butschlii and the intestinal flagellates often stain surprisingly well with them. If flagellates are present it is rather a good plan after staining with Leishman or Giemsa to dip the film for half a minute or less into 1% iodine and then wash : brings out the flagella, but rather spoils the chromatin staining of nucleus.

C. Hæmalum. Mayer. (Dobell's formula). Hæmatoxylin crystals 1 gramme, distilled water 1,000 c.c. Dissolve, and add Potash alum 50 gms., Sod. Iodate 0. 2 gramme. Solution should be rich red. Does not keep, and if any precipitate present discard. Take film from distilled water, pour on stain, stain 5 to 10 minutes. Now wash in running tap water till the slide turns blue : (expose to sunlight whilst doing so, or, if a cloudy day add a drop of Liq. ammoniæ to washing water). Hæmalum gives a good general ripe picture of the intestinal protozoa and cysts, and is the easiest rapid stain. It fails to bring out nuclear detail. It stains E. coli and E. histolytica well.

The REAL method, however, of getting good permanent preparations of intestinal protozoa is Schaudinn fixation followed by full Haidenheim's Iron hæmatoxylin technique, (next lecture). Above A, B, C, methods are only suitable for film preparations made for purposes of confirming saline and iodine findings.

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LECTURE 4.

E. HISTOLYTICA, CONTINUED. LAB DIAGNOSIS OF ENTAMŒBÆ E. GINGIVALIS.

During acute amœbic dysentery stools contain active, motile, vegetative E. histolytica. They are not infective: even if motile E. histolytica was swallowed it would be killed by gastric juice. It is the stools of the convalescent and healthy carrier which are the source of danger.

In amœbic abscess of liver pus is sterile, because the histolytica are sterile. E. histolytica not found in pus from abscess, the living ones are away in liver abscess walls burrowing into fresh tissue. They may sometimes be found in pus draining away from walls of abscess on 3rd or 4th day after open operation. N.B. This, incidentally, is the place to look for binary fission forms. E. histolytica never encysts in the liver, would simply defeat its own objects in life if it did.

As patient recovers the residual E. histolytica still go multiplying in colon wall: but fæces become more solid. Importance of recent work at Calcutta on pH, (as a measure of alkalinity or acidity) of stools. Find that the pH for active, motile E. histolytica is very constant indeed, usually 6.24, whether in kitten gut, kitten passed stool, human amœbic stool full of motile histolytica. E. coli shews wide pH range, and so does E. nana, from 5 to 8. Intestinal flagellates found in alkaline stools, at pH range of about 7.5 to 9. Probably the impetus to encystment of E. histolytica, and possibly of all intestinal parasites, is change in pH of stools as patient recovers.

Anyhow in convalescent amœbic case and in histolytica carrier, as the motile, vegetative E. histolytica come out from colon wall into lumen of gut and down it, they encyst.

Pre-cyst of E. histolytica, " E. minuta," or minuta phase, is smallest form of the amœba concerned. All ingested R. B. Cs. thrown out, pseudopodia withdrawn, entamœba becomes tiny, round, motionless ball with exceedingly clear endoplasm.

Pre-cyst of E. histolytica usually smaller than that of E. coli; but only differential point is that it retains histolytica type of nucleus. Nucleus is large relative to cyst, as it is about to divide. Cyst wall is thinner than that of E. coli. Pre-cyst 5 to 14 μ in diameter: three races in size with average diameters of 8, 11½ and 13½ μ.

As cyst wall secreted traces of glycogen may be seen, but never a big glycogen vacuole, as in E. coli. In at least 27% —probably more—of histolytica cysts chromatoid bodies are formed early and are very prominent. Here note (a) some persons always pass small, others always big histolytica cysts: (*b*) in some persons almost every cyst passed shews splendid chromatoid bodies, in others very few or no cysts shew them. Chromatoid bodies of E. histolytica cysts are the most prominent feature of the cyst: stand out in saline as bright, hyaline, refractile rods, bars, chunks. In Iodine not well seen. When stained with Iron hæmatoxylin stain jet black. Represent plastid or food reserve substance secreted by nucleus and laid down in cytoplasm as reserve.

Nucleus divides into two, then four. Histolytica shews a wider range of cyst types than E. coli: 53% of cysts seen in stools are adult 4 nucleate. If a stool is found full of 4 nucleate cysts it is either E. nana or E. histolytica, and these are easily distinguished from one another.

2 nucleate stage of cyst shews 2 nuclei, no glycogen or merest trace, prominent chromatoid bodies. 4 nucleate adult cyst shews 4 nuclei, in each instance nucleus true to histolytica type, with fine central karyosome and thin chromatin deposit on nuclear membrane, and cyst nuclei are invisible in fresh state: cf. E. coli for contrast. Carrier state diagonosed by finding 4 nucleate adult cysts in formed stools.

Puzzle as to how emetine cures amœbic dysentery. In therapeutic doses has no action whatever upon either motile or encysted histolytica. (The only entamœba which really IS affected by emetine is I. butschlii, and this infection in man can be wiped out by one single hypodermic dose of emetine). In 1 in 1000 concentrations added to fresh amœbic stools motile E. histolytica moves about and enjoys itself under microscope. Probability is that emetine either alters pH of environment and renders it unsuitable, or else emetinises the R. B. Cs., whereupon the E. histolytica die out from starvation. No doubt as to the great therapeutic value of the drug. But not always successful: and we especially find that the smaller strains of histolytica are the most resistant.

E. histolytica infection acquired by swallowing cysts. Importance of flies, and above all of cooks, massalchies etc. as carriers. Fly visits latrine and then dinner table bringing cysts on his pads, cyst will even pass unaltered through fly's gut. Human carrier infects food supplies from his hands, or fæces.

Exactly what happens when cyst swallowed not known ?? does one amœba, or do four amœbæ creep out. Cyst passes unchanged through the stomach, probably excysts in small intestine. Penfold, Woodcock and Drew (1916) placed one part of centrifuged deposit from an emulsion of cysts in medium :—Broth 5 parts, Bengers' Liquor pancreaticus 2 parts. Incubated 5 to 7 hours at 37°C. Saw cyst wall dissolve, contents flow out en masse. They failed to secure excystation of cysts of E. coli and of Giardia in same medium.

Cutler (1919) adopted similar method, but first exposed cysts to pepsin : said a single protoplasmic mass emerged from cyst and divided into four amœbulæ. These experiments were repeated by Dobell and Stevenson with negative results.

Yoshida, 1918-1920, claims to have watched both encystment and excystation of E. coli and of E. histolytica, but his plates suggest processes of degeneration rather than of life. He describes fusion of nuclei (autogamy) in the mass of protoplasm which emerges from the cyst. Cutler claims to have grown E. histolytica on two media as follows :—

Egg medium. White and yolk of an egg thoroughly broken up by shaking in a glass bottle containing beads. Add 300 c.c. distilled water and shake thoroughly. Bring gradually to boil on a water bath and boil for half an hour, keeping contents constantly shaken. Get a fluid with egg particles in suspension. Put into 5 c.c. volumes in test tubes and autoclave.

Blood clot medium. Take 500 c.c. of clotted human blood and boil for one hour in a litre of water. Filter and add 0.5% NaCl and 1% peptone. Tube and sterilize by steaming for 20 minutes on each of three consecutive days.

To both media a few drops of fresh blood should be added before inoculating. To inoculate get a fresh stool full of blood, mucus and motile E. histolytica. About 5 or 6 large loopfuls added to 5 c.c. tube of medium plus fresh blood. Temperature of 28 to 30°C, better than 37°C., as it keeps down bacterial contamination. Examine and subculture very frequently, daily for preference.

Very doubtful whether Cutler obtained true cultures, as apart from mere survival of the entamœbæ in his cultures.

A study of the pH environment may lead to discovery of suitable artificial medium of right pH for histolytica cultures, when much experimental work will become possible, including investigation of action of different drugs on it.

Classical experiments of Walker and Sellards, Philippines, 1913. As in E. coli experiments selected 20 men free from all intestinal parasites, and similar precautions as regards food and drink as in the E. coli experiments. Gave coated capsules containing E. histolytica cysts by mouth. 18 out of the 20 become parasitised by E. histolytica, and 4 of them went on to suffer from amœbic dysentery, at intervals of 20, 57, 87 and 95 days respectively, (probably a measure of their different relative resistance).

Best present day mode of culture of E. histolytica is in the colon of young kittens. Either :—(*a*) feed kittens on fæces full of adult 4 nucleate viable E. histolytica cysts : about 50% take and go down with amœbic dysentery. Or (*b*) take fresh amœbic dysentery stool full of motile vegetative E. histolytica, make emulsion, with rectal tube inject very high up into colon. Here about 90% or more take and the strain can be passaged from cat to cat. Cats usually get acute amœbic dysentery of classical type, but chief symptom of all is great emaciation. Colon is ulcerated and gut contents and stools full of motile histolytica with included R. B. Cs. But have once seen a curious condition where cat died of extreme emaciation : on p.m. colon found quite unaffected but cæcum severely ulcerated and full of motile E. histolytica, cat never having shewn clinical dysentery. At Calcutta four strains successfully established, but in each instance a natural coccidiosis of cats set in, probably a cage infection, the pH of the stools become very acid, about 4.4, and histolytica infection died out. Cysts are never seen in cats : emtine has no action on amœbic dysentery in cats : disease invariably fatal, incubation 2 to 6 days, on injection per rectum.

Many workers on experimental amœbic dysentery in cats. Hlava, 1887 : Kartulis, 1887 infected kittens per rectum with liver abscess pus : cats developed amœbic dysentery. Several cases in literature of cats experimentally infected with amœbic dysentery developing liver abscess, and similar case of badger in Kartulis' lab. Puppies less susceptible but James records finding Indian pariah dog infected in nature, and question may be raised whether pariah dog , who must often eat fæces full of E. histolytica cysts, may not act as carriers of the infection. Guinea pig also susceptible but amœbiasis in this animal an entierly different disease: colon not ulcerated, infection settles in cæcum, tremendous lymphoid reaction, sort of pseudo-tumour forms and proves fatal. Work well worth investigating ?? in man is there ever limited amœbiasis of the cæcum without clinical dysentery, ?? also, has amœbiasis any relationship to carcinoma of the colon, cæcum and rectum ?? Chronic amœbic dysentery in man often followed by visceroptosis.

Lab. Diagnosis of Entamœbic Infections. N.B. ALWAYS INSIST ON FRESH MATERIAL FOR STUDY.

1. First record macroscopic appearance of stool. Bacillary dysentery disease often in in-patients, far the commoner: as patient at once flattened out by fever and toxæmia. Amœbic dysentery is disease of out-patients and is typically WALKING dysentery. Bacillary dysentery stool milky white, blood bright red, mucus whitey yellow, alkaline to litmus, pH about 8 to 9. Amœbic stool diarrhœal, or even semi-formed, grey to green, fæcoid with blood and mucus adherent to and intimately mixed with fæces. Blood dark reddish brown, much of the HB changed to acid hæmatin. Usually acid to litmus. pH almost always exactly 6.2. (N.B. Note importance of pH in prognosis: if in semi-cured patient the pH of the stool has gone up to 8 or 9 conditions are unsuitable for histolytica, and cure may be sound: if still at 6.2 still suitable for the parasite, and betting is the case will relapse).

2. General microscopic characters of stool in saline emulsion. Bacillary stool richly cellular: field full of cells. Full of R. B. Cs. which are unaltered and in rouleaux and shew up well. Crammed with leucocytes, some 90% of them polymorphs. Much mucus in streaks. Intestinal epithelial cells with big flat nuclei. Macrophages: i.e. phagocytic cells, either from endothelium of capillaries, wandering plasma cells, or large hyaline mononuclears. Amœbic stool different. Cellular content scanty. R. B. Cs. crenated and shew up badly. Leucocytes less numerous. Use of 1 in 600 fluorescein in the saline good: it stains all dead cells, e.g. macrophages, which may simulate entamœbæ.

3. Next search for entamœbæ. Safest rule is not to diagnose any vegetative entamœba unless you see it moving about. Having found one, which is it ?? Here a word of caution. We may find E. coli moving about in a bacillary dysentery stool: don't on this diagnose amœbic dysentery. (*b*) We may find cysts of E. histolytica in a person who is a histolytica carrier, but whose current attack of dysentery is

due to bacillary causes. (*c*) We may be dealing with a case of mixed dysentery : i.e. bacillary plus amœbic, when both motile E. histolytica with ingested R. B. Cs., and dysentery bacilli will be found. In reality ALL DYSENTERY STOOLS SHOULD BE PLATED AS WELL AS MICROSCOPICALLY EXAMINED. Recent pH work however suggests that mixed dysentery is a misnomer : that where we get both histolytica and dysentery bacilli, it is a phase of transition of the pH, either bacillary supervening on a previous amœbic infection, or amœbic infection supervening on a bacillary onset.

Remember that any entamœba with included R. B. Cs. is E. histolytica. In all cases the stool, whatever its consistency, should be examined both in saline and in iodine.

4. The finding of Charcot Leyden crystals is of great assistance. Are rhomboidal, lozenge-shaped, hyaline and translucent crystals, from 10 to 70 μ in size. Acton's recent work seems to shew that they are always associated with amœbic, and never with bacillary dysentery. They are found, both during the acute amœbic dysentery phase (probably this means a relapse in a carrier) and often long into convalescence when no entamœbæ can be found. Chemically they appear to be ethylimine and a product of histolytica digestion of tissues (cf. their presence in autolysed tissue in asthma sputum). A finding of Charcot Leyden crystals is quite enough to indicate emetine treatment.

5. Never give a diagnosis of "No amœbæ present : " say "No entamœbæ seen." It's up to the clinician to make what he can of this.

6. Where we are looking for cysts and for the carrier state we are usually dealing with a formed stool. Again, however, go through above 5 steps. In addition grind up a portion from the centre of the stool in 1% iodine : centrifuge heavily : throw away supernatant fluid, and emulsify particle of deposit in saline under cover slip. This gives stained cysts against colourless background. Objects in stools which simulate entamœbic cysts : see plate by Knowles and Cole in Indian Jl. Med. Research, Jan. 1917, and illustrations in Cammidge's "Fæces of Children and Adults." (Note : the views as to E. coli, E. histolytica as "E. coli communis" in the paper by Knowles and Cole have now long been abandoned, but the state of the literature in 1916 was enough to justify the paper at that date).

7. Use of cyst counts to determine degree of severity of infection. Emulsify one gramme of fæces in 10 c.c. of iodine

solution. Centrifuge. With leucocyte pipette of hæmatocytometer cell pick up to 0.5 mark of deposit and dilute with saline to 11 mark=1 in 20 dilution. Fill Thoma Zeiss hæmatocytometer cell and count all cysts seen in total ruled area. This gives total number of cysts in—approximately—0.5 c. mm. of stool at 1 in 20 dilution, whence total number of cysts per gm. of stool can be roughly estimated. A special counting chamber for protozoa devised by Cropper is on the market. Vide Proceedings Royal Society of Medicine, 1918, Vol. XI, General Reports, page 1.

8. Several methods have been recommended for concentrating entamœbic and other cysts in stools. The following is recommended by Mackie :—

(*a*) Emulsify the fæces thoroughly in 10% formalin saline.
(*b*) Filter through wire gauze.
(*c*) Filter through muslin.
(*d*) Centrifuge at speed 1,800 per minute for one minute.
(*e*) Dilute up the deposit with solution :—Citric acid 12 gms. formalin 2 c.c., distilled water 100 c.c. (Specific gravity 1,047). Add on the top 2 c.c. of ether.
(*f*) Centrifuge for 30 seconds.
(*g*) Break up the film between the ether and the fluid below to set free any cysts entangled in the ether.
(*h*) Centrifuge again for 30 seconds.
(*i*) Throw off the supernatant fluid with a sudden jerk and examine the deposit for cysts.

Doubtful value of cyst concentration methods. All of them are laborious and they do not much improve the percentage of positive findings.

9. Valuelessness of negative findings. Wenyon took an R. A. M. C. hospital personnel. 92 apparently healthy men examined. 12 found to be passing histolytica cysts but from 2 to 6 daily examinations were necessary before all 12 had been discovered.

10. Laboratory diagnosis of amœbic dysentery by post. Quite easy. If acute amœbic dysentery be suspected smear out films of blood stained mucus on slides. Either send dry or better send fixed in spirit, for staining and examination. If carrier be suspected emulsify portion of formed stool in 1% iodine, and send emulsion by post for examination. No use sending the stool itself : will be decomposed on receipt.

6. *Entamœba gingivalis.* First entamœba discovered. Gross. 1849. Literature again very confused and all sorts of different amœbæ described from the mouth. Dobell only admits one and says other descriptions are inaccurate accounts

of the same amœba. Smith, Barrett and Johns claim that it causes pyorrhœa. No proof whatever that it does so has yet been put forward. All animal experiments negative. Small entamœba, rather like a minature E. coli : 10 to 20 μ. Very actively motile however. Pseudopodia round and lobose. Little ectoplasm. Cytoplasm packed with vacuoles, bacteria, food particles, and nucleus usually hidden from view by them. Also in endoplasm lie peculiar greenish, round, hyaline masses in food vacuoles. Mistaken by many workers for included R. B. Cs., but Dobell shews that they take nuclear stains, are not R. B. Cs., and are probably merely ingested food particles in process of digestion. Nucleus not conspicious in fresh state : is typical entamœbic ring nucleus : well marked prominent central karyosome, around this clear space, then ring-like deposit of chromatin on inner surface of nuclear membrane.

Multiplies in vegetative state by binary fission. Cyst not yet discovered (it must have one of course). How infection acquired not known. Much further study wanted.

Lives in tartar on teeth, also found especially in pus of pyorrhœal pockets, and any oral pus. Similar entamœba in cats and dogs. Mitchell and others found E. gingivalis in 22 % of mouths of healthy children : in 24 out of 42 persons with no pyorrhœa : but in 38 out of 40 with pyorrhœa. Increased association with pyorrhœa, however, may simply mean that pyorrhœal state affords better feeding ground for this entamœba. Dobell considers it perfectly harmless and even useful commensal and of value as a scavenger.

"**The Full Iron Hæmatoxylin Method.**" (Haidenheim).

It is exceedingly hard to get good, permanent preparations of the intestinal protozoa. The ONLY really efficient method is Dobell's, as follows :—

1. Make a thin emulsion of PERFECTLY FRESH stool. With a platinum needle spread as a thin even smear on a cover slip. A slide may be used instead and is easier to handle but means use of much more fluids. To prevent cysts etc. from washing off either spread a thin smear of egg albumin fixative or of fresh serum on cover slip. This keeps cysts etc. in situ but does not improve staining. It is really better omitted.

2. Wait till the film is JUST on the point of drying. Before it dries place it very gently (so as not to wash off the film) in Schaudinn's fixative : absolute alcohol 1 part to 2

parts of a saturated solution of chemically pure corrosive sublimate in normal saline, and with 5% glacial acetic acid added to it. Schaudinn's fluid is best used at 60°C, but may also be used cold. 10 to 20 minutes will completely fix.

3. Rinse in 50% alcohol and transfer to 70% alcohol tinged a port-wine red with iodine crystals dissolved in it. The iodine changes the mercury to mercuric iodide. Keep in this till the film is well browned.

4. It is at this step advisable to leave the preparation for 12 to 24 hours in rectified spirit or absolute alcohol to harden. In any case the brown mercuric iodide must now be removed by soaking the film in rectified spirit till it is white.

5. Pass through the graduated alcohols, 70%, 50%, 30% to distilled water.

6. After soaking in distilled water the film is now ready to stain. Soak for 3 to 4 hours in a saturated watery solution of Iron alum crystals. The iron alum MUST BE THE PURPLE VIOLET CRYSTALS : the green stuff is oxidised and useless. This mordants the film.

7. Rinse very quickly in distilled water. Now stain with " ripe " hæmatoxylin. To prepare get hæmatoxylin crystals—(Grubler's gives far better results than any other) : make a 0.5% solution and expose to sunlight in a flask plugged with cotton wool till it is a rich brown red : may take some weeks. Stain for 6 hours or more. The film should be subjected to several changes of stain, until it turns a jet black.

Or Shortt's carbolized Hæmatoxylin may be substituted. Dissolve one gramme of Hæmatoxylin crystals in 100 c.c. distilled water. Bring to the boil. Add 5 c.c. pure carbolic acid and allow to cool. This may be substituted for the " ripe " hæmatoxylin above.

8. The Iron Hæmatoxylin process is a " regressive stain " : the slide being first uniformly over-stained and the stain then extracted. Wash the film in distilled water and place it in 1% iron alum solution (of the violet crystals). THIS IS THE MOST IMPORTANT STEP IN THE PROCESS. The iron alum gradually removes the stain and gives a differential picture, leaving chromatin stained a jet black but cytoplasmic structures unstained. From time to time rinse with water and examine the film under the microscope to see how differentiation is proceeding. Vegetative amœbæ may decolourise in 3 to 10 minutes, cysts may take hours and even require a 2% to 4% iron alum solution. The moment the right differentiated picture is obtained :—

9. Wash thoroughly, half an hour, in tap water.

10. At this stage the film may be counterstained by immersing it for half a minute in 1% Bordeau red (to stain cytoplasm), but this is not necessary. If counterstained wash again.

11. To dehydrate pass successively through 30% alcohol, 50% alcohol, 70% alcohol, rectified spirit to absolute alcohol and soak in the last. Absolute alcohol is never absolute in the tropics : to make absolute powder copper sulphate crystals, desiccate by strongly heating over a Bunsen till you get a white dry powder : cool : put this in the absolute alcohol : it dehydrates the absolute alcohol, and should be changed when it turns green.

12. Soak in equal parts absolute alcohol and xylol. Transfer to xylol.

13. Mount the film in neutral (non acid) Canada balsam.

14. The film should never be allowed to dry at any step of the procedure.

This process is long, tedious and troublesome : it is however the only way in which good permanent preparations can be got. The results in a good slide amply repay all the trouble expended. In use for fæcal films Leishman and Giemsa stains may sometimes give a good picture but usually do not : hæmalum gives a ripe but not differentiated picture and is useful for rapid diagnostic work : but the full iron hæmatoxylin method is THE ONE PAR EXCELLENCE. It alone stains the flagella of intestinal flagellates and is the only stain which shews detail in cysts. It is too laborious and expensive for general class use, but is well worth it for anyone who desires good permanent preparations.

Remember to mount under or to use a No. 1 (or thinner) cover slip : the oil immersion lens will not focus through a thicker one.

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MORPHOLOGY OF THE HUMAN ENTAMOEBAE (after Dobell and Wenyon).

	ENTAMŒBA COLI.	ENTAMŒBA HISTOLYTICA.	ENDOLIMAX NANA.	IODAMŒBA BUTSCHLII.	DIENTAMŒBA FRAGILIS.	ENTAMŒBA GINGIVALIS.
VEGETATIVE FORMS, **Size.**	20 to 40 μ. Never less than 10 μ.	20 to 30 μ. Sometimes less than 10 μ.	6 to 12 μ.	9 to 15 μ.	8 to 9 μ.	10 to 20 μ.
Motility.	Sluggish.	Very active if fresh.	Sluggish.		Very active.	Active.
Ectoplasm.	Scarcely seen.	Well seen. ⅓rd of parasite.	Scarcely seen.	Well seen in motile amœba.	Very clear.	Scarcely seen.
Pseudopodia.	Blunt : big : rounded : consist mostly of endoplasm.	Thin finger-like if fresh : later are lobose. Consist only of ectoplasm.	Blunt.	Blunt : consist of clear ectoplasm.	Fragile : leaf like. When moving amœba resembles a snail.	Round : lobose.
Colour and appearance in fresh state.	Greenish. "ground glass."	Glassy : clear : yellowish.	Greenish.	Very refractile.	Fragile.	Greenish.
R. B. Cs.	Are never ingested.	Any Entamœba containing ingested R. B. Cs. is E. histolytica.	Never ingested.	Never ingested.	Never ingested.	Never ingested : but the greenish hyaline masses may look like them.

Endoplasm.	Densely granular: packed with bacteria, yeasts, debris of all sorts. Voracious feeder.	Finely granular. Shows only included R. B. Cs. or tissue cells. Dainty feeder.	Full of food particles. Mulberry-like Sphærita often seen.	Full of bacteria and food particles.	Granular but fragile. Contains bacteria and food particles.	Full of bacteria : vacuoles : and hyaline greenish masses and leucocytes.
Bacteria.	Are freely ingested.	Are never ingested : but parasitise the dying amœba in stools.	Are ingested.	Are ingested.	Are ingested.	Usually packed with bacteria.
Vacuoles.	Often numerous : usually seen as clefts or cracks in endoplasm.	Scanty : spherical : defined.	Many : well seen.	Numerous.	Often present.	Crammed with vacuoles.
Nucleus (unstained).	Conspicuous as a clear, large, bright round or oval ring.	Usually invisible.	Scarcely seen : a dull blob.	Not seen.	Two nuclei : well seen.	Not conspicuous.
Nucleus (stained).	Coarse type with much chromatin. Big, excentric karyosome : halo around : clear space with chromatin grains : thick ring deposit of chromatin on nuclear membrane.	Delicate type with scanty chromatin. Central small karyosome : halo : clear space free from chromatin : thin ring deposit of chromatin on nuclear membrane.	Blob like. Much chromatin. Chromatin mass in bizarre forms inside nuclear membrane.	Large. Much chromatin. Big central nucleolus : clear zone with chromatin dots : nuclear membrane well defined.	Two nuclei alike. Nuclear membrane well seen. Big central star shaped cluster of chromatin.	Well marked central karyosome : halo : clear space : ring like thick deposit of chromatin on nuclear membrane.
Multiplies by	Binary fission probably. (Not yet described).	Binary fission only. (Dobell and Wenyon). (only seen in gut wall).	Probably binary fission.	Probably binary fission.	Not known.	Binary fission only.

	ENTAMŒBA COLI.	ENTAMŒBA HISTOLYTICA.	ENDOLIMAX NANA.	IODAMŒBA BUTSCHLII.	DIENTAMŒBA FRAGILIS.	ENTAMŒBA GINGIVALIS.
PRE-CYST.	"Minuta phase," Spherical: clear: single nucleus. 12 to 17 μ. Three races in size.	"Minuta phase," Spherical: clear: central nucleus. 5 to 14 μ. Four races in size.	Very small 5 to 8 μ. Very clear and transparent. Single large nucleus.	Thick cyst wall. Volutin grains and iodophilic body forming.	Not known. (It rapidly becomes very vacuolated and degenerated when passed in stools).	Not known.
ENCYSTMENT.	Glycogen vacuole often very prominent, especially at 2 nucleate stage. Chromatoid bodies very rare; only in 5 % of cysts. 4 nucleate phase rarely seen.	Glycogen vacuole indistinct and soon disappears. Chromatoid bodies seen early. Coarse and highly refractile. Nucleus relatively large to cyst	Straightforward nuclear multiplication to 2 and 4 nucleate stages. Glycogen vacuole and chromatoid bodies rare.	" Iodine cyst."	Not known.	Not known.
ADULT CYST.	Usually spherical. 8 nucleate.	Spherical. 4 nucleate.	Often oval. 4 nucleate.	Quite irregular shapes. 1 nucleate.	Not known.	Not known.
SIZE.	15 to 22 μ. Three races.	6 to 18 μ. Four races.	About 8 μ.	9 to 12 μ.		
GLYCOGEN.	May be faint traces.	Has disappeared.	None.	Deeply staining iodophilic mass.		

Chromatoid Bodies.	Very rare : only seen in 5 % of cysts. Fine feathery : like "splintered glass."	Big : coarse : and most characteristic. Seen in 27 % or more of cysts.	Occasionally seen : filamentous.	None. Big refractile volutin granules however.		
Nuclei.	"Coli type": (as above).	"Histolytica type": (as above).	The 4 nuclei are often clustered together near one pole.	Ex-centric : in rim of cytoplasm around iodophilic mass. Chromatin massed ex-centrically on inner aspect of nuclear membrane.		
Frequency.	87 % of E. coli cysts seen in stools are 8 nucleate.	53 % of E. histolytica cysts seen in stools are 4 nucleate.	4 nucleate forms by far the most common.	Cysts often found in stools in grouped masses.		
Variations.	16 nucleate big cysts.	8 nucleate cyst ? (Very doubtful).	8 nucleate cyst has been noted.	None : except in size and shape.		

LECTURE 5.

THE MASTIGOPHORA. NATURE OF THE FLAGELLUM. FLAGELLATA. INTESTINAL FLAGELLATES. BLASTOCYSTIS.

Mastigophora an immensely important group in tropical medicine. Include parasites of kala azar, oriental sore, espundia, trypanosomiasis, Chagas' disease. Universal distribution of flagellate protozoa. Many thousands of free living species. Hundreds of species parasitic in reptiles, insects, animals and some in man. Even plants infected. Pathogenicity varies from harmless Bodo of lizard to deadly trypanosomes of sleeping sickness. Intestinal flagellates, infection transmitted by resistant cysts in fæces, contaminative. Blood inhabiting flagellates infection transmitted by biting insects and leeches. Which came first, the trypanosome of sleeping sickness in man's blood or its form in the gut of the tsetse fly ?

Mastigophora so called because they possess one or more flagella. Flagellum a long, thin, fine, whip-like, vibratile lash of protoplasm. Often longer than body of protozoon. Shape of mastigophoron constant, has definite cell membrane, but is elastic and compressible, can bend round corners and over obstacles and move either backwards or forwards.

Flagellum arises from nuclear apparatus. Best studied in the binucleate flagellates = Order Binucleata. Trypanosome, e.g. has two nuclei, big macronucleus = vegetative, trophonucleus : and towards the posterior pole of cell a small, deeply staining micronucleus = kinetonucleus. Macronucleus concerned with cell nutrition : micronucleus with cell motility and cell division. Use and misuse of term blepharoplast. If we go back to diagram of typical cell structure we find :—(*a*) nucleus (here micronucleus) ; (*b*) centrosome ; (*c*) archoplasmic vesicle, here in flagellates represented by eosinophile vacuole close to micronucleus ; (*d*) basal granule of nucleoplasm from which flagellum arises. Strict definition of a blepharoplast = centrosome related to motor apparatus : but often the micronucleus is inaccurately called a blepharoplast. In reality whole of this apparatus is reproduced at root of flagellum.

But centrosome is within micronucleus : hence we can only distinguish micronucleus, vacuole and basal granule. On other hand traces of flagellum root may be traced towards macronucleus = rhizostyle. Where mastigophoron has only one nucleus the flagellum arises in relation to it. Flagellum itself consists of central core of nucleoplasm (part from cell membrane to nucleus = rhizoplast), prolonged like central wire of electric wire to tip. This sheathed by a covering of cytoplasm, like rubber sheath of electric wire. Axial filament and cytoplasmic sheath. Like whip with stock handle. In Mastigophora cell division starts at centrosome, i.e. within the micronucleus : then macronucleus divides, then flagellum splits longitudinally into two flagella : finally macronucleus and cell cytoplasm divide, and cell divides, always longitudinally from anterior to posterior pole into two. Usual pattern = binary longitudinal fission.

Complication in books and discussion about true polarity of the flagellates. Compare diagrams in Castellani and in Manson. In most flagellates flagellum projects in front of cell : is horse which pulls the cart : or like aeroplane propeller tugging aeroplane after it. But in Trypanoplasma the main flagellum projects behind cell, like propeller of steamer or tail of spermatozoon, urges flagellate forward in front of it. The flagellum always arises from a basal granule. In most Mastigophora it passes forwards, free in Herpetomonas, united to the body by an undulating membrane in Crithidia and Trypanosoma. In Trypanoplasma however the main flagellum passes backwards instead, united to the body by an undulating membrane. Accept Manson's and not Castellani's diagram. Actually both Herpetomonas and Trypanosoma can move either forwards or backwards.

There may be more than one flagellum. If so we always find that all of them arise from basal granules at anterior or posterior pole of cell ; and always one main, thicker principal flagellum, with one or more subsidiary, and often trailing flagella. (N.B. Don't speak of flagellæ : they belong to the bacteria, not protozoa).

Huge and complex classification of this big group of the Mastigophora. Main sub-groups (*a*) Euflagellata or flagellates proper, which include all human species. (*b*) Dinoflagellates with thick, cellulose cuticle, two flagella, one in ventral groove, one free : most of them marine forms, many with calcareous deposited exo-skeleton. (*c*) Cystoflagellata, which have cyst like bodies immensely distended by gelatinous substance : often luminous : e.g. Noctilua="jelly fish" of Indian seas. Only concerned here with Euflagellata.

Sub-classification of Euflagellata. (*a*) Pantostomina : have pseudopodia as well as flagella : capture food at any part of body surface : are free living forms in salt water. (*b*) Protomonadina. One principal flagellum plus one or more subsidiary flagella. Immense group, including very many free living forms, and in connection with man the genera Enteromonas, Bodo, Embadomonas (or Waskiá), Prowazekia, Cercomonas, and all Hæmoflagellates : hence of great importance. (*c*) Polymastigina, with three or more unequal flagella. For man includes Trichomonas, Chilomastix and Giardia (vel Lamblia). (*d*) Euglenoidina : large and very complex forms : free living : very common in pond water etc., with mouth, œsophagus, colour spots, vacuolar system etc. (*e*) Chromomonadina : small free living forms with green or brown colour spots or chromatophores. (*d*) Phytoflagellata, with nutrition holophytic, like that of plants, green colour, all free living, e.g. Gonium. All human genera come into (*b*) and (*c*) above : but this shews how small a part they are of this immense protozoal sub-kingdom.

For convenience sake and cutting right across systematic classification we may divide flagellate protozoa of man into (1) intestinal flagellates, and (2) blood inhabiting flagellates= Hæmoflagellates=Order Binucleata, (human species of the Binucleata i.e.).

Human Intestinal Flagellates.

Inhabit fluid fæces of small or large intestine and even may adhere to mucosa wall, e.g. Giardia. Literature on them now just starting to become as muddled as entamœbic literature : new species described almost every month. Again for the sake of reason and safety accept Dobell's classification, until further evidence brought forward. Very many so called intestinal flagellates are accidental visitors to the intestine or bedpan. Are seen especially in diarrhœic stools with high pH in motile state, e.g. pH of 8 to 9, e.g. in bacillary dysentery (hence mistaken notion that they may cause dysentery). In formed fæces we see only their infective cysts. Giardia the only one with any **REAL** claim to pathogenicity.

Here again Dobell clearly recognises (1) Coprozoic flagellates, merely accidental flagellates from outside world with special liking for living in fæces : i.e. forms which either pass as cysts through the intestine and then excyst and grow in the passed fæces or get into the stool from the water used to wash the bedpan : and (2) true parasitic intestinal flagellates of man.

Coprozoic Intestinal Flagellates.

Apart from the very rare Copromonas (common in stale fæces of frogs) and Helkesimastix, each of which has been once cultivated from stale human stools by Dobell (and were obviously derived from ærial or water contamination), we may recognise three clearly defined genera. There are probably many other coprozoic flagellates : but these are the chief ones :—

1. *Cercomonas.* (Many books say this is a very common intestinal flagellate of man, but that is because many laboratory works call every flagellate they see Cercomonas). Actually it is rather uncommon. Oval, about 6 μ long, flattened on one side as if it lay like a leaf on paper. Single nucleus with big karyosome. One principal flagellum arises at anterior pole, and passes free, directed forwards. Other arises at same point, directed backwards, adheres to body of protozoon, projects free behind as trailing flagellum. At anterior pole where the flagella arise protozoon is drawn out into sharp point. Food ingested by sort of amœboid process. Multiplies by longitudinal binary fission. Forms spherical, 1 nucleate cyst full of bright refractile granules : cysts 4 to 6 μ in diameter. Two species : C. longicauda with small karyosome, very long anterior flagellum, and very short posterior flagellum : C. crassicauda with bigger karyosome and much shorter anterior flagellum. Cercomonas is identified in fresh preparation by its slow rotatory movement, like that of a dying Catherine wheel firework.

2. *Bodo.*—Genus Bodo is a very big one. Many free living Bodo forms. Many parasitic forms : gut of almost every lizard shews a Bodo-like flagellate, B. lacertæ. Narrower and longer body than Cercomonas. Big vesicular nucleus with big karyosome near middle. At anterior pole is a big blepharoplast and basal granule. Traces of a cell mouth or cytostome also present, and a small contractile vacuole at anterior end. Narrow ovoid shape. Two flagella arise at anterior end from the big basal granule="kinetoplast" of Alexieff. One short stumpy one passes forwards, other long, slender, trailing one passes backwards and projects free behind. Special tendency for short anterior flagellum to stick to any particle in saline emulsion : contracts as it does so : whence Bodo has a peculiar jumpy jerky, back and forward movement when seen in fresh state. Two species at least coprozoic in

human stools. B. caudatus, 10 to 18 μ, (bigger than Cercomonas): lanceolate or leaf shaped, with small, oval, cyst 5 to 7 μ: with single nucleus, kinetoplast, and some deeply staining granules. B. edax smaller, 6 to 14 μ long, stumpier than caudatus, conspicuous snout over cystostome, big kinetoplast. Third variety, B. urinarius, Hassall, 1859, needs confirmation. In reptiles Bodo is a very frequent and true intestinal flagellate, but in human stools it is almost certainly merely coprozoic. Its jumpy movements identify it.

3. *Prowazekia.* This genus is of considerable systematic importance. Dobell considers it = and is identical with Bodo. Others differ. Several species described, P. cruzii in Brazil., P. asiatica in Ceylon and Burma. P. parva free living in damp earth. P. weinbergi in Tonkin. P. muscæ, in gut of house fly (=probably a mistake for H. muscæ domesticæ).

Recently in Calcutta found one in the mouth and saliva of a case of splenomedullary leukæmia: case swarming with an oral Prowazekia, and easily cultivated on Row's medium= accidental contamination of mouth, in no way pathogenic. Prowazekia usually described as just like Bodo, but (*a*) in place of "kinetoplast" is a big, definite micronucleus: and (*b*) flagella are different, anterior long; posterior thick and stumpy. Cyst 6 to 7 μ long.

Here importance of ascertaining whether Bodo is 1 or 2 nucleate. Dobell insists Prowazekia=Bodo, and "micronucleus"=kinetoplast. Calcutta Prowazekia has anterior structure so large and prominent that it deserves name true micronucleus. If Prowazekia is true genus with 2 nuclei then very interesting as it would constitute a transitional stage between Bodo and the Binucleata. Position still ill defined.

These three genera are, in man, purely coprozoic, and not true parasites. Are easily cultivated at any temperature, at almost any pH, on almost any nutritive media. What appear to be truly parasitic similar genera are found in abundance in gut of reptiles, frogs, snakes, lizards etc. where conditions probably favour their growth and establishment. Only importance to us is that they occur in some one to three per cent. of human stools: especially in stale, diarrhœic ones.

True Human Intestinal Parasitic Flagellates.

These are different, and are truly parasitic in intestine, though even then mostly harmless commensals, like the harmless entamœbæ. Recognise here five clear genera; Embadomonas (or Waskia) : Enteromonas (or Cercomonas) : Trichomonas : Chilomastix (or Tetramitus) : Giardia (or Lamblia).

1. *Embadomonas* (or Waskia). Only species E. intestinalis. Wenyon, 1917. Very rare. Wenyon had a case in Egypt with stools full of it. Patient in next bed contracted infection and got even heavier infection. Tiny and unimportant flagellate. Very minute, 5 μ long or less. Single, big anteriorly placed nucleus with small karyosome and a few chromatin dots. Just behind nucleus is big depression = cell mouth or cytostome. Two flagella : one long, thin, passes forwards : other short, thick, stumpy, lies partly in cytostome, party extruded. Similar jerky movement as with Bodo. Long anterior flagellum causes forward movement, short stumpy posterior one jerks. Divides by longitudinal binary fission. Cysts very minute, pear shaped, 5 μ, and inside shew shadow outline of cytostome and a long deeply staining looped thread = posterior flagellum. Only importance of this parasite is that it may be discovered in India any day. Quite harmless. Has peculiar characteristic when seen in fresh state : comes up to and rests sideways on under surface of cover slip : so that with its anterior bulging pole projecting over its mouth it looks like a bird on a twig.

2. *Enteromonas* (Cercomonas). Here systematic position very doubtful. 1. Very small, oval flagellate described under several different names. 4 to 8 μ long. Single, big vesicular nucleus near anterior pole, with big karyosome. Ordinary variety (= Tricercomonas of Wenyon) has 4 flagella. 3 arise friom anterior basal granule and pass forwards free : 4th passes back as delicate trailing flagellum. Divides by binary longitudinal fission. Cysts are oval, elliptical, 7 μ long : at first contain only one nucleus : this then divides into two : then into 4, placed in two pairs : one pair at each pole. Two species described : one with only one anterior flagellum = Monocercomonas : one (as above and usual one) with 3 anterior flagella = Tricercomonas. But the cyst etc. are so different from coprozoic Cercomonas (vide above) that it is better to regard it as a different genus, Enteromonas, with two species, Mono- and Tri-enteromonas. Literature here very confused indeed. We pass however, next to

The Three Really Important Intestinal Flagellates of Man.

3. *Trichomonas.* Relatively common human intestinal parasite : 1922 Calcutta figures give it present in 18 out of

233 stools from 185 patients in hospital. Discovered (but described as "Cercomonas") by Davaine, 1854 in stools in enteric fever. Two MOST important points about it are (*a*) its cell wall is very thin, very liable to deformity and distortion: and (*b*) it possesses a clear undulating membrane. Intestinal variety, T. hominis, is small and active flagellate, 7 to 20 μ, but usually about 10 to 15 μ long. Shape rounded but ends in a finely drawn out, pointed tail. Near anterior pole is big, oval, single vesicular nucleus with big, central karyosome, and a few chromatin extra granules. At anterior pole is an ill defined group of basal granules. Three flagella arise anteriorly and pass forwards. 4th, big, thick and very prominent flagellum arises at same spot, i.e. anterior pole, but passes back along body of protozoon: united to it by undulating membrane, which=raised up cell envelope plus cell cytoplasm: passes to pointed posterior tail tip of parasite and then projects for a short distance free behind. This is the most prominent flagellum of the lot. As it lashes about it throws the undulating membrane into wavy folds, whence the name undulating membrane. Undulating membrane displays a continuous rippling motion, and appears—owing to being placed laterally—to pass from one side to other of parasite, as the parasite turns over. Trichomonas has an aimless sort of movement. It swims first in one direction, then in another, then off again in another direction and so on, like the gambols of a puppy.

From the basal granule group arises a stiffening vertebral column rod, as it were, of nucleoplasm=axostyle. Passes over and around the big nucleus to the posterior pole of cell and acts as a stiffening rod or sort of vertebral column. Stains feebly. At anterior end is also a small crescentic slit=cytostome, but not very prominent.

Trichomonas divides by binary longitudinal fission, and may sometimes be seen in division, (such specimens have been described as a new "Octomitus" parasite by several workers). Cysts of T. hominis have never been discovered or described: (most accounts of so called Trichomonas cysts are really Blastocystis hominis). Cysts of the very common and similar T. batrachorum of frogs are well known.

When seen in fresh stool Trichomonas easily identified by undulating membrane, small size, pointed, sharp, stiffened tail, and puppy dog like movements. But soon after stool is passed it starts to degenerate. It now shews a quite different and VERY CHARACTERISTIC APPEARANCE. The undulations persist: but the form is constantly changing in shape, It assumes a sort of pseudo-amœboid activity. As its cell membrane is so delicate and as it becomes nearly motionless we see a long thick, finger like protrusion (just like the thick tail of the L.D. body—vide later) form and pass down the body of the parasite. This was mistaken by Castellani for "Entamœba undulans," which=dying T. hominis. Appearances in stale stool very characteristic.

Trichomonas a very common parasite. Wenyon's figures, 3% in 550 persons examined : in 14 out of 393 cases with fluid stools. Several varieties described : (*a*) intestinal varieties with 4 and 5 in place of three anterior flagella=Tetra—and Penta-trichomonas respectively. Chatterji described Pentatrichomonas in stools of 32 consecutive cases of dysentery in Calcutta : but no controls, no proof that they were not bacillary dysentery cases where the stools are often full of flagellates, no control 32 non-dysentery cases either. Also big variety, T. vaginalis, described in vagina in cases of leucorrhœa with acid reaction, and T. buccalis from the mouth.

Several workers claim to have cultivated Trichomonas. Boyd, 1918-1919, recommends :—Loopfuls of fæces containing Trichomonas are transferred to normal saline tubes and incubated at 37°C. After 10 days loopfuls of sediment are transferred to fresh tubes containing unsterilized emulsions of fæces from persons free from flagellate infections. After the second transference the Trichomonas multiply. After 18 days subculture into similar tubes. Ohira and Noguchi cultivated T. buccalis in diluted ascitic fluid. Chatton (1920) cultivated Trichomonas of the guinea pig in a medium of meat bouillon mixed with rabbit blood. Such cultures however most difficult to obtain and to keep alive. Probably for success a p*H* of about 7·84 is requisite.

No real evidence to connect Trichomonas with disease Wenyon however has found it lying deeply in sections of the mucosa and considers that it may have some claim to pathogenicity. As with all the intestinal flagellates Trichomonas feeds voraciously, ingesting bacteria, yeasts etc. It also ingests R. B. Cs. readily if present in a dysenteric stool, but this is no proof of its pathogenicity. Similar Trichomonas an extremely common parasite of gut of frogs and reptiles : who also shew a very similar Trichomastix : which differs from Trichomonas only in one point : the 4th, posterior, trailing flagellum is not attached to the cell body of an undulating membrane, but is free, and lashes with a long, pendulum like movement.

4. *Chilomastix* (or *Tetramitus*) *mesnili*. Again discovered by Davaine in cholera cases, 1854, and again misdescribed as a Cercomonas. Very common, perhaps commonest intestinal flagellate. Splendid monograph on it by Chalmers, 1918. Annals Trop. Med. and Parasitology. Clearly described first by Wenyon, 1910. 3 % incidence in fluid stools of cases ; 1922 Calcutta figures give it present in 22 out of 233 stools from 185 patients. Known to be world wide in its distribution. Similar species in baboons, apes, fish and lizards.

Differs from Trichomonas and is easily identified by :—(*a*) Much larger size, 10 to 15 but sometimes 20 or more μ ; (*b*) very stiff and unyielding cell membrane. When motile Chilomastix (and Giardia) die in stool they assume no such pseudo-amœboid forms as does Trichomonas : they simply die in situ and shew dead fixation images ; (*c*) mouth apparatus, which is prominent ; (*d*) no undulating membrane ; (*e*) most characteristic movements in motile state. When in fresh stool it swims actively forward and especially tends to stick to solid particles in the emulsion. A little later it has a slow, bumpy, oscillatory movement like that of a barge tied up to a pier head in a flowing stream, bumps to and fro. Later still dies and is non motile.

Body shape rigid, and not infrequently shews a sort of spiral twist upon itself. Big oval, vesicular nucleus near anterior pole with linin network studded with chromatin granules, sometimes a small excentric karyosome. At anterior pole diffuse group of blepharoplasts = basal granules. 3 equal flagella arise from these and pass anteriorly forwards. Also fourth posterior flagellum, which lies in the cytostome.

Most prominent feature of parasite is its huge cytostome or cell mouth. Begins as a big, wide groove with thickened lips, supported by thickening fibrils, and passes back obliquely across the body of the parasite. In it lies the 4th, posterior flagellum. In fact Chilomastix uses its lips with which to eat its food and its 4th flagellum with which to lick its lips. This 4th flagellum is the biggest and most prominent of the lot.

In motile state Chilomastix divides by longitudinal binary fission. Its cyst, found in formed stools, is most characteristic structure. Is small, 7 to 8 μ (about size of an R. B. C.), exactly like a lemon in shape. May contain a small lump of glycogen. Inside is the shadow outline of parasite : i.e. basal granule, lip of cytostome groove, nucleus and 4th flagellum, latter well seen in stained preparations.

Chilomastix very often seen in diarrhœic stools. Similar parasite in Egyptian rats ?? same or different species ?? Chalmers considers it is like E. histolytica : sometimes pathogenic, usually not. No real evidence that it is.

Chilomastix lives in the upper part of the colon : possibly also in the lower part of the large intestine. Boeck (1921) has succeeded in cultivating it. Medium used :—Sod. chloride 0·9 gm, Calcium chloride 0·024 gm, Potass. chloride 0·042 gm, Sodium bicarbonate 0.02 gm. Dextrose 0.25 gm, distilled water 100 c.c. Sterilize by steaming. Add one part of human or horse, or sheep serum to four parts of above. Incubate tubes overnight at 37°C before inoculating. Inoculate with loopfuls of mucus or fæces containing motile Chilomastix washed through three changes of sterile saline. Incubate at 37°C. Make frequent, almost daily subcultures. Boeck kept strain alive for 4½ months. Knowles finds average pH associated with motile Chilomastix in stools to be 7.40, probably most suitable pH for cultural attempts.

5. *Giardia* (*Lamblia*) *intestinalis*. The most important of the lot. Here we have a parasite with at least some claim to pathogenicity. Most workers admit that whilst (1) it is often present in health and not associated with disease : yet (2) it is often the cause of intractable diarrhœa, even ??? of dysentery. Position probably like that of E. histolytica. Lives in small intestine, especially jejunum (whereas other flagellates live in cæcum and colon). Found by Knowles as apparently sole cause in sprue, diarrhœa cases and in k.a. dysentery (cultures negative). World wide distribution. Calcutta 1922 figures, in 21 of 233 stools from 185 k.a. cases. Wenyon found it in 5% of healthy troops. Lamblia rarely seen motile in stools, and the vegetative form when seen in diarrhœic stools is usually dead. Has a slow tumbling movement of progression. Like Chilomastix has very rigid body envelope. Flat leaf like shape. Bilaterally symmetrical and leaf like form. Vegetative form (usually seen dead and a little degenerated in stools) looks like a pear cut across, or a monkey face, or a gas mask. Convex and concave surfaces. On concave surface is saucer-like sucking disc by which it adheres to cells of jejunal mucosa. Sucking disc has raised margin. Tail sharply pointed, like a paper kite. Two big, oval nuclei, anteriorly placed, each with big cluster of karyosomes. Length 10 to 18 μ. Nuclei look like eyes with pupils. Between nuclei lie two parallel bars= fused rows of basal granules. From them arise 4 pair of flagella (Lamblia belongs to the Octomitidæ with 8 flagella). Front pair pass forwards, cross, like hair combed over temples. 2nd

pair like moustaches project laterally. 3rd pair are most prominent of lot, and leave cell at lower margin of sucking disc like beard or whiskers. 4th pair project behind like "imperial." In centre of parasite is a chromidial mass, (better seen in Giardia of rat than in Giardia of man). Vegetative form multiplies by binary fission. Whole complex nuclear and flagellar structure reproduced on dorsum of parasite, which then divides into two new ones. (Like cutting leaves of unopened book).

As it passes into lower part of small intestine Giardia encysts. MOST peculiar process. Cysts found in formed stools. Have clear, jelly like cyst wall. About 14 μ long, oval : inside lies greenish parasite a little shrunken away from cyst wall. Early cyst shews shadow outline of parasite : near anterior pole both nuclei : paired rods of basal granules, rudiments of 3rd pair of flagella. Next this one Giardia divides into two (note how extremely unusual this is). Two new Lamblias come to lie head to tail inside cyst : note that adult typical Lamblia cyst shews OBLIQUE LINE OF SEPARATION BETWEEN THEM. This absolutely characteristic. If cyst is still in jejunum this cyst now ruptures, setting free two new motile Lamblias (note again how extraordinarily unusual this is). Usually cysts passed in formed stool : infect new host. If cyst be seen end on it looks round, but line of division of the two Lamblias can usually be made out, or other typical oval cysts seen from side. Also in stale stools especially "blue Lamblia cysts" are often seen : small, 7 to 10 μ, staining slatey blue with iodine ?? glycogen converted to starch. Thus we may find one of three varieties of cysts in formed stools :—(*a*) degenerated minute blue cysts ; (*b*) early cysts with only one individual, glycogen and two nuclei at anterior pole ; (*c*) adult cysts with two Lamblias inside and typical oblique line. Lamblia diarrhœa is a disease which it is practically impossible to treat : usually intermittent. No worker has yet succeeded in cultivating Lambia. Knowles' 1922 findings of average pH of 6.09 when vegetative forms present : cysts found in both acid and alkaline stools.

There may be other intestinal flagellates of man, both coprozoic and parasitic : but NOT ONE OF THEM HAS YET PROPERLY DESCRIBED, IDENTIFIED OR PLACED UPON A PROPER SYSTEMATIC BASIS. The literature at present is getting almost as muddled as that of the entamæbœ for 1900-1914.

Blastocystis hominis, is extremely common in human stools, especially in starchy and fermentative stools. Nature is unknown but it is almost certainly fungoid and not protozoal in nature. Size VERY varied, from 5 to 50 μ. Is a bladder like cell, consisting chiefly of an enormous central vacuole filled with "cellulose jelly" (which often shews crack-like fissure-like markings). Either on one or both sides lies a rim of cytoplasm studded with from 2 to 6 little bright, refractile nuclei. Resemblance of one rim forms to engagement ring. Young forms shew less vacuole, much rim cytoplasm, nuclei well seen. Old forms are reduced to little more than a huge bladder filled with cellulose jelly and little if any rim. It divides (*a*) by binary fission. (*b*) Wenyon describes a formation of innumerable Blastocysts inside an enveloping membrane as if multiple fission within a cyst. (*c*) Acton draws attention to giant forms (which much resemble vegetable cells from the diet) splitting up to form typical Blastocystis. When present in stool usually present in enormous numbers, seen in almost every field. Barrett, 1921, cultured in 10% human serum with 0.5% saline, (but gives no figures). Undoubted true intestinal and exceedingly common parasite of man. (Calcutta 1922 figures in 66 out of 233 stools from 185 patients). Is NOT, as previously thought either the cyst of Trichomonas, or a degenerating leucocyte, but is probably fungoid in nature and origin. Some workers describe multiplication by budding.

In general to study intestinal flagellates adopt methods as for entamœbæ. Iodine will shew up flagella. In making stained preparations it is often useful to add a loopful of fresh blood to the stool emulsion : smear : stain by Leishman : wash : then expose for half a minute to Gram's iodine, which brings out the flagella.

Students should from now on take every opportunity to examine all available stools for both entamœbæ and flagellates.

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LECTURE 6.

BINUCLEATA, CRITHIDIA, HERPETOMONAS, LEISHMANIA. THE LEISHMANIASES.

Hartmann, 1907, proposed to class all blood inhabiting Mastigophora in Order Binucleata, as they are all binucleate. ?? should Prowazekia be included ??. Binucleata are order of immense importance, include many parasites of man, animals, fish, insects, reptiles, birds, even plants. Vary from perfectly harmless to deadly parasites. Characterised by having two nuclei : (1) micronucleus, governs cell movements and cell division and stains deeply : present as small dot or bar of chromatin : (2) macronucleus, governs cell nutrition and stains less well. All have one chief flagellum, one genus —Trypanoplasma, has second flagellum. Classification :— into :—

1. *Genus Crithidia.* Macronucleus central, micronucleus just in front of it, anterior flagellum starting from near micronucleus runs forwards, traces of an undulating membrane.

2. *Genus Herpetomonas*, (vel Leptomonas). Macronucleus central, micronucleus anterior, single anterior flagellum.

3. *Genus Leishmania.* Either identical with or very closely related to (2). Rounded non-motile forms in tissues of man and dogs. Macro- and micro-nucleus. In culture become herpetomonads.

4. *Genus Trypanosoma.* Macronucleus central, micronucleus at posterior pole. Flagellum arises from basal granule just in front of micronucleus at posterior pole and passes forwards along body, united by undulating membrane, projects free in front of the cell.

5. *Genus Trypanoplasma.* Macronucleus central or near anterior pole. Big, huge micronucleus close to anterior pole. Chief flagellum passes back to tail of parasite, united to it by an undulating membrane, but also second flagellum arises at same point but passes anteriorly : i.e. heteromastigote arrangement as in Bodo.

6. Should Prowazekia come in here as extra genus ?

Crithidia. Occur in gut of both biting and non-biting insects. Many so called Crithidias are really developmental stages of trypanosomes. Chironomos, water beetle, Tabanidæ etc. infected with. Resemble (2), but micronucleus close to macronucleus, and flagellum traverses cell.

Herpetomonas. Immensely important genus. Transitional between intestinal and hæmo-flagellates. Life cycle of H. culicæ, Patton, Scientific Memoir 57. May be taken as typical. Great importance of these in working at k. a. transmission, may be mistaken for developmental forms of L. donovani. Imperative necessity in working on k.a. of using insects lab. bred, known free from natural herpetomonad infections. Gut of infected Culex larva shews small, round, oval forms, 3 to 4 μ diameter, round macro- and tiny rod-like deeply staining micronucleus, (Leishmania form). Divide in gut by binary fission : clusters of dividing forms result. Culex larva becomes adult mosquito. Parasites grow, develop into flagellate forms, midgut finally packed cram full with actively motile rosettes of flagellates. Divide by longitudinal binary fission : flagellum, micronucleus, macronucleus divide in turn. Rosettes of wriggling flagellates with flagella all towards centre, interlocked. Note tendency in Mastigophora of all sorts to colony formation. Flagellates 15 to 25 μ long by 3 to 4 μ broad. Feed on food particles, cytoplasm often stuffed with them. Fagellates stick to gut wall. Pass into rectum of mosquito : here lose flagellum, change back to Leishmania forms, passed in fæces as cyst-like, ? resistant forms : infect water : infect new generation of larvæ. Summary : pre-flagellate, leishmania phase in larva : adult flagellate phase in adult insect midgut : post-flagellate stage in mosquito's rectum = infective form. Infection by contaminative cycle of Minchin.

Herpetomonas of Euphorbia plants probably = herpetomonads of the hemiptera bugs feeding on them. Infected Euphorbias found in compound of a k.a. hospital, probably accidental occurrence.

H. ctenocephali of dog flea, Ct. canis of interest. Many workers notably Laveran, Franchini, Fantham etc. have shewn that injection of such insect flagellates into mammals produces disease resembling k.a. with spleen and viscera full of L.D. bodies, (Leishmaniasis in the making). Thus herpetomonads of water beetle or of sheep ked when injected into white mouse produce k.a.-like disease with L.D. in viscera : (but this could not occur in nature). Flagellates of fleas, dog fleas, rat fleas, anopheles mosquitoes, melophaga flies etc. (all biting) produce such diseases on culture and experimental injection. View held by Patton, Rogers, Laveran, Fantham etc. that k.a. parasite in evolution = herpetomonad of a biting insect. But other vigorous opponents of this view, and later workers have failed on careful repetition of experiments to confirm them Insect herpetomonads cause little or no inconvenience to their insect hosts. Best material for experimental study is the green " bottle fly," Lucilia, of which some 90% shew a natural herpetomonad. H. muscæ domesticæ of common house fly another good example. ?? are the Leishmania parasites herpetomonads in evolution ?? are trypanosomes ditto, and in some instances such as T. lewisi of the rat which causes no symptoms, has not only insect transmitter, but also vertebrate host become acclimatised to them ?

Genus Leishmania. (Ross.).

Patton now refuses to recognise this genus and calls the parasites of k.a. and oriental sore Herpetomonas donovani and tropica respectively.

Genus *Leishmania* (separated by Ross from Herpetomonas) comprises :—

1. *L. donovani*, parasite of Indian kala azar.
2. *L. infantum*, parasite of Mediterranean kala azar.
3. *L. tropica*, parasite of oriental sore.
4. *L. tropica*, American variety, cause of Espundia.
5. Leishmania parasite which causes canine leishmaniasis of dogs. To which we may add (6) is Sudan k.a. parasite of Archibald another species or not ? First question is :—?? are these all one and the same or different parasites ?? Appearance, morphology, cultural forms practically identical. Yet oriental sore and k.a. very different diseases, geopraphical distribution very different, also L. tropica, but not L. donovani, can live in a septic environment. In India take line from Cape Comorin to Almora : then all k.a. lies east of it : all oriental sore west of it. In Mediterranean basin where both infantile k.a. and oriental sore exist together, k.a. is found in sea ports, oriental sore in desert villages. K.A. a disease of sea ports and riverine areas, oriental sore a disease of desert regions.

Row, 1914, got local nodules in skin of monkeys injected with L. donovani. Laveran and others have produced generalised and fatal k.a.-like disease, especially in white mice, from injections of L. tropica. Is oriental sore localised leishmaniasis, and k.a. visceral and generalised leishmaniasis, both due to same parasite ? But, on the whole, difference clear : and best plan is to admit three varieties :—

1. L. donovani : parasite of Indian k.a. Varieties, Sudan and Mediterranean parasites. 2. L. tropica, parasite of oriental sore, variety S. American parasite. 3. Parasite of canine leishmaniasis.

Leishmania donovani.

Simultaneously discovered, 1903, by Leishman and Donovan, whence name. Parasite found in k.a. in all internal viscera, especially spleen, liver, bone marrow. Also in peripheral blood.

Peripheral blood in kala azar. First Patton found parasite in finger blood of 90 % Madras cases. (Note. Personally I believe that possibly the reason why the Madras workers find L.D. in 90 % of peripheral blood films is that they wont do spleen puncture and are therefore driven to go on examining peripheral blood films of suspected cases until they DO find the parasite)....Earlier workers in Assam failed : later Mackie got 20 % positive films, later Knowles at Shillong got 40 % positives. 1922 Calcutta figures shew 20 % persons positive : 118 k.a. cases (diagnosed as such by spleen puncture) : 409 films : 24 cases and 50 films positive. 1 to 15 parasites per positive film found. KALA AZAR CAN ALMOST ALWAYS BE DIAGNOSED BY EXAMINING PERIPHERAL BLOOD FILMS OFTEN ENOUGH AND SUFFICIENTLY CAREFULLY : (yet thousands of patients ? k.a. ? malaria in Bengal are exposed to long and not entirely safe antimony treatment on purely clinical diagnosis). Peripheral blood findings perhaps improved by giving half to one c.c. adrenalin half an hour before taking films. Technique for films :— Take 6 to 12 GOOD films with good leucocyte edge : examine with low power, (best is $\frac{1}{7}$th oil immersion), examine LEUCOCYTE EDGE ONLY AND RAPIDLY. Waste of time to examine any other part of film.

As met with in peripheral blood L. donovani is found :— (1) in the polymorphonuclear leucocytes. Here it stains badly and is clearly being phagocytosed ; (2) in cytoplasm of both large hyaline mononuclear leucocytes and of capillary endothelial cells met with free in blood. Here it stains well, dividing rosettes are seen, parasites are very viable, and ?? does large mononuclear function rather as host cell than as phagocytic to the L.D. body ?? ; (3) very occasionally free in blood plasma, where also dividing forms may be found.

Apart from finding of parasites in peripheral blood films, culture of the peripheral blood is ALWAYS POSITIVE IN KALA AZAR. Where for any reasons spleen puncture is to be avoided, peripheral blood culture is THE method of diagnosis. Either :—(1) take 2 c.c. of blood and sow on to 6 to 9 N.N.N. tubes : or (2) take one c.c., add to 10 c.c. of citrate saline, keep overnight in cool incubator : next day sow deposit on N.N.N. culture medium. Culture may take 21 days to become positive. B. M. Das Gupta got 33 out of 35 Assam cases positive on peripheral blood culture (the two negative ones had had a lot of antimony and were well on the way to cure) and in 1922 in Calcutta 23 consecutive cases positive on peripheral blood culture.

THE PARASITE OF KALA AZAR CAN ALWAYS BE RECOVERED FROM THE PERIPHERAL BLOOD EITHER ON DIRECT EXAMINATION OF FILMS OR ON CULTURE.

General blood picture in k.a. worth noting from point of view of subsidiary value. Anæmia steadily deepens, till counts of one million or even less R. B. Cs. are recorded. HB. falls proportionately : to 20% or even less. Total leucocyte count is PERSISTENTLY LOW e.g. down to 2000 per c.mm. or less. (Total leucocyte count in malaria may be low, but rises and falls). Personally, from Assam cases regard the following blood picture as characteristic of k.a. rather than of chronic malaria :—1. total leucocyte count persistently below 4000 per c.mm. ; (2) polymorphs, in differential count less than 50% (are worn out in phagocytic struggle with parasites) ; (3) large mononuclears more than 20%. In chronic malaria any two of above may be present, but rarely, if ever, all three together. McVail draws attention to cases of hookworm infection with low eosinophile percentage : often = kala azar. Word of warning : marked leucocytosis may occur in kala azar, especially with sepsis, cancrum oris, broncho-pneumonia, etc. Case seen in child 3½ years old in Assam : total leucocyte count 74,500 per c.mm., and 2512 parasites in 12 consecutive blood films examined. Presence or absence of parasite in peripheral blood appears to be purely accidental : bears no relationship to time of day or night, state of disease, habits of biting insect etc., not periodic : here today, gone tomorrow in films.

L. DONOVANI IS AN INTRACELLULAR PARASITE. Lives in cytoplasm of endothelial cells throughout body, spleen, bone marrow, liver, lymph glands, pancreas, testes or ovaries, intestinal mucosa etc. Dysentery of k.a. appears to be either amœbic or bacillary, and not due to k.a. parasite. Parasite kills by inches, by wasting of essential tissues. Destruction

of endothelium replaced by fibrosis : fine fibrosis of essential organs, e.g. heart muscle, pancreas, kidneys : strangulation of tissue, atrophy, thus flabby heart (special tendency to sudden death in k.a.). Spleen films shew parasite (1) in immense numbers in endothelial cells ; (2) in large mononuclears ; (3) free ; (4) in polymorphonuclears where, again, undergoing degeneration. Leucopænia of the disease is due to wearing out of polymorphs in phagocytic struggle.

L. donovani ovoid, oval or bean shaped parasite, 2 to 4 μ diameter. Definite and very marked cell envelope and contour, (important point in diagnosis from platelets etc.). Plano-convex shape. Pale blue cytoplasm with Romanowsky stains. Large, round macronucleus on or near convex border. Micronucleus usually deeply staining, rod-like bar or fine, deep staining dot, usually placed at an angle to the macronucleus. Multiplies within endothelial cells by binary fission, producing schizogony rosettes of 2 to 64 individuals. Shape and size of L.D. varies very much. On spleen puncture we may meet :—(1) ordinary big forms ; (2) especially where only blood and not pulp withdrawn " torpedo forms " of Knowles. Tiny, oat-shaped forms, (??? motile, Mrs. Adie), always seen free and not in cell, with micro- and macro-nuclei very closely approximated. These are very viable, and give good cultures. ?? are they L.D. in transit from cytoplasm of endothelial cell in blood stream before entry into leucocyte ??

Spleen puncture will diagnose at least 97 % of cases with certainty. Perfectly safe operation : carried out on several thousands of patients by Napier, Mackie, B.M. Das Gupta, Knowles. Idiotic idea that it is dangerous. Shillong technique. Patient prepared beforehand. Night before (adult) given 30 grs. calcium lactate : if any suspicion, do blood coagulation time, and don't puncture if over 4 minutes, (usually is about 3¼ minutes) ; but have done cases with 5½ minutes. Next morning half an hour before puncture 30 grs. calcium lactate. Get patient ready. Syringe (Roux pattern much the best) must be sterile and dry (e.g. if boiled in water wash out with normal saline or parasites will be hæmolysed). Under patient, placed flat on sofa with hands behind head, pass binder. Have abdominal pad ready. Have adrenalin and pituitrin ready : though have never had occasion to use either. Paint iodine over spleen : puncture at most prominent point. Assistant FIXES SPLEEN UP AGAINST RIBS WITH HIS HANDS SO THAT IT DOESN'T MOVE. Go straight in. (Napier recommends two movements : personally go right in at once). Withdraw plunger with strong suction : (here a Roux is worth ten times an Arnold). Withdraw sharply. Instantly abdominal pad and binder applied : patient kept on his back 30 minutes, last 30 gr. dose of calcium lactate given. Use a fairly big needle. Whole question of risk depends on how long one is in the spleen : for culture leave a little saline in syringe : for films use dry syringe. Absurd statement in books that liver puncture is safer : one could do splenectomy for hæmorrhage of spleen, but how control severe hæmorrhage from the liver ?? Occasional cases of k.a. with non-palpable spleens, especially noted by Dodds Price : here do liver puncture : torpedo forms especially seen here.

Methods of culture of L. donovani parasite : applicable to peripheral blood, spleen juice, p.m. spleen, bone marrow etc. (1) Rogers used sodium citrate saline, slightly acidulated with citric acid. 1904. (2). Usual medium. N.N.N. (originally introduced by Novy, Nicolle and McNeal in Sleeping Sickness Commission in C. Africa for trypanosomes, whence name). Agar, 14 to 20 gms., salt, 6 gms., water 1000 c.c. Sterilize, melt on water bath at 50°C. Add to each tube when liquid 2 to 3 c.c. of fresh rabbit blood, obtained by direct cardiac puncture of rabbit. Slope to solidify and set. When inoculated parasite grows and flagellates in water of condensation at bottom of tube. Do 6 to 10 tubes per inoculation, as some may go septic and spoil results. (3) Row recommends taking peripheral blood into citrate saline for 24 hours before sowing tubes, to dilute antibodies, see above. Row's medium is especially useful for subcultures. Take 10 c.c. of rabbit blood : defibrinate by shaking in sterile bottle with glass beads. Add 10 volumes of distilled water to lake the blood. To prepare medium add one volume of this laked and defibrinated blood to two volumes of 1.2% NaCl solution.

L. donovani (whether in N.N.N. culture or in the bed bug) assumes a motile, herpetomonad, flagellate form under several well defined conditions, viz :—1. temperature must be between 22 and 28°C : i.e. cool incubator : higher temperatures gradually immobilise and destroy flagellates ; 2. HB. must be present ; 3. oxygen needed : in anærobic conditions flagellates simply die out by degrees ; 4. slightly acid medium best : Napier prefers N.N.N. acidulated with HCl : pH about 6.4.

Rogers' discovery of 1904. In culture from spleen juice infected cells at once disintegrate. L.D. parasites set free. Become larger and vacuolated. Start to divide rapidly by binary fission. Swarm produced of small rapidly dividing forms. Pale eosinophile vacuole=archoplasmic vacuole, now appears in front of micronucleus. Inside it appears rhizoplast of future flagellum. By degrees parasite elongates, becomes much larger : is converted into typical HERPETOMONAS. Young immature flagellates appear about 3rd day in culture, (Napier reports them in 24 to 48 hours in acidulated N.N.N.), by 8th day tube swarming with flagellates. Flagellates divide by longitudinal binary fission : produce rosettes of motile flagellates with intertwined, centripetal flagella. Grow in water of condensation of N.N.N. tubes. **EXACTLY SIMILAR TO INSECT HERPETOMONADS EXCEPT THAT SEPSIS IN THE CULTURE KILLS THEM OUT.**

Later, according to Row, as culture gets old, flagella disappear : L.D. forms round up, become Leishmania-like again : start dividing and smaller and smaller post- and super-post flagellate forms are produced. Row claims that these constitute the most infective forms of the parasite ?? Very doubtful whether Row's post-flagellates and super-post-flagellates are really such : or whether they are not merely L.D. bodies which have not developed, and degenerated L.D. flagellates : even Row does not describe the characteristic cell envelope or capsule which the true L.D. post-flagellate should show.

Flagellates survive freezing. Sepsis in the culture kills them almost at once. By subculturing on 7th to 14th day strain may be kept going indefinitely in cool incubator. Laboratory strain kept going by Laveran in Paris for 12 years. Napier obtained flagellates as early as 48 hours on acid N.N.N., usually appear about 4th day : by 8th day water of condensation of tube swarming with flagellates : this the time to subculture. Sometimes development delayed 3 weeks, ? even longer. In old cultures very small, non motile, ? degenerate leishmania forms seen. Row insists on post- and super-post flagellate stages, which, he says are extremely infective to white mice. Archibald in Sudan describes coccoid, granule-like forms. Cornwall doubts all these post-flagellate forms. Note that all above five conditions needed for development of the flagellate are present in the midgut of Cimex. Leishman describes such active longitudinal binary fission that ultimately very thin, almost spirochæte like forms produced. **B. M. Das Gupta at Shillong in one set of drying up N.N.N. cultures got pucca, but curious new phase of the L.D. flagellate. Flagellates suddenly became encysted inside thin, membraneous like cyst wall. Inside this pseudocyst it divided into swarm of daughter flagellates. These formed wriggling mass inside cyst, trying to get out : with flagella all pointing centrifugally : cf. and contrast ordinary rosette : appearance like man inside a sack. Then cyst burst : 2nd generation flagellates set free and swam** away : these are very fine, stain very deeply, very viable. ?? is this phase of encystment part of extra-human cycle or is it merely reaction to unfavourable environment (drying culture). Whole process took only 24 hours : next day the cultures shewed adult flagellates, ruptured cysts, small active 2nd degeneration flagellates. ?? can L. donovani ever encyst ?? are encysted forms ever passed in fæces.

Forms seen in cultures then are flagellates, post- and super-post or aflagellates, cysts (only seen once).

TRANSMISSION OF KALA AZAR

How does kala azar pass from man to man ? Today the most outstanding and interesting unsolved problem in tropical medicine.

Here note interesting suggestion (suggestion merely) by Wenyon. It is obvious that L. donovani is—essentially—a herpetomonad : and presumably an insect herpetomonad. Suppose now that in a given area some insect very heavily infected with this herpetomonad : regular epidemic of this herpetomoniasis in some insect in locality. Man occasionally bitten : gets herpetomoniasis=kala azar. This would mean that there is no transmission at all : that accidental kala azar in man is simply end phase of an insect epidemic. Plague is a disease of rats, not of man. Is kala azar herpetomoniasis of some insect, man being accidentally infected as an end phenomenon, and no transmission from man to man at all ? Interesting speculation, but whole epidemiology of the disease is against it. Assam workers' evidence that cure of early cases in villages stops spread of epidemic is evidence against this view. Presume at least that kala azar is essentially a disease of man, presumably transmitted from man to man.

If so, note second further possibility. Napier notes how, although k.a. fever and temperature chart very erratic, yet two clear types of onset especially, (*a*) typhoid-like, even sometimes with positive Widal. Some of Napier's recent cases are "cured" enterics from the Medical College Hospital. L.D. in spleen. (*b*) chronic malarial type, with rigors, and simulate malarial closely except for progressive splenic enlargement. Hence arises a question : in a k.a. district is it possible that almost the whole community get infected with herpetomoniasis, but that only such persons as contract secondary diseases, enteric, malaria (malaria and k.a. DO UNDOUBTEDLY CO-EXIST IN SOME PERSONS), amœbic or bacillary dysentery, etc., go down with pucca k.a. ?? In other words ARE THERE

LATENT KALA AZAR CARRIERS, persons carrying L. donovani, but without symptoms ? Believe there are : half cured antimony cases for one : secondly cases such as Mackie's : man walking about for years with big spleen, no fever L.D. plus at several punctures ; Knowles' case, child dying of k.a., father subacute k.a., mother didn't know she was ill or had a big spleen, spleen L.D. plus. Further question ?? is half treatment of cases not in hospital, especially cases in private practice converting k.a. cases wholesale into k.a. carriers ??

To sum up, however, must admit k.a. specific disease of man due to L. donovani. Must regard the k.a. case as source of possible infection of others. K.A. is a SITE AND FAMILY DISEASE.

First question is " How does the parasite escape from the infected patient ? ". Cultures and examinations of urine, saliva, sputum negative. Does it leave by fæces ? Knowles has spent 5 years looking for L.D. in fæces. Mackie, Knowles, and others describe " cystic bodies " in k.a. fæces. But general examination of fæces in k.a. shews that they are like fæces of non kala azar. ONE point however : 55% of k.a. cases shew stools crammed with yeasts as against only 10% of non kala azar. Yeasts a very striking feature of stools of k.a. patients, ? fermentation. Believe cystic bodies are not L.D. at all, but are yeasts. Further work by Christophers and Knowles on ankylostome as possible transmitting agent : results after much work entirely negative. L. donovani may possibly pass from man in the stools, many Assam doctors strongly favour theory of soil contamination : but many years of hard work have never shewn any recognisable form of L.D. in k.a. stools. K.A. dysentery is like non kala azar dysentery, due to E. histolytica or dysentery bacilli. Nasal mucus examined occasionally : negative.

Does parasite leave via skin ulcers ? Claim made by some workers that L.D. present in skin ulcers. But bullæ examined, cancrum oris lesions examined, none found. K.A. not especially associated with skin lesions at all. Extraordinary twocases, 1922, of dermal leishmanoid : a new disease and new from of leishmaniasis. Histories of both cases similar : patient got k.a., spleen plus L.D., even peripheral blood plus L.D. one case. Patient put on to antimony treatment. Cured. At some time or another during or just after treatment rash appears due to outside cause, in one case syphilis, in the other ? antimony rash. Fades. But parasites present in peripheral blood settle in rash spots : patient gets cured of his k.a., spleen small, fever gone, blood picture that of health. Yet L. donovani settles (from peripheral blood) in rash spots : goes on to produce soft granulation tissue : finally we have healthy person, free from all k.a. symptoms, with spleen and peripheral blood cultures even negative : but covered from head to foot with lesions looking like leprosy (nodular) but due to L. donovani, *i. e.* dermal leishmanoid. Cultures of nodules positive : of peripheral blood negative. Yet such cases are rarities. Ordinarily L.D. NOT found in skin lesions of k.a., and believe the parasite does NOT escape via skin.

Believe as working hypothesis at least that parasite leaves via the peripheral blood. Evidence in favour of this : parasite found in at least 40% of cases if sufficient films examined, Peripheral blood cultures of untreated cases always positive. If so, then parasite must leave patient via a biting, non-flying, localised, blood sucking insect.

Turn to other end of chain. How does parasite get into man ? Subcutaneous inoculations of flagellate cultures at least very rarely if ever take. Experimental monkeys infected by heavy intraperitoneal injection. This not natural channel. Shewn recently by Gupta and Knowles that oral infection cannot be put out of court. Archibald claims to have infected monkey by mouth, feeds of spleen juice. Cornwall had one white rat : fed on spleen juice : several weeks later cultures of heart blood positive : later still killed, examined, no infection found. Calcutta 1922 monkey. Fed on both 22nd and 23rd May on fresh k.a. post mortem spleen : meal washed down with 30 c.c. of thick spleen emulsion by stomach tube. Liver puncture and cultures negative, 11th July, 1st August. Brought into laboratory in dying state, 25th November. Emaciated, suffering from dysentery. Chloroformed and post mortemed. Viscera, spleen, (four times normal size and fibrotic) full of L.D., bone marrow, kidneys, even heart blood shewed L.D. Monkey literally died of k.a. with typical symptoms.

May seem quite absurd, yet is suggested for consideration that cycle of k.a. transmission may be quite simple. No need to postulate post- or super-post-flagellate stages. Suppose some biting insect X in close association with man picks up L.D. from patient's peripheral blood. L.D. flagellates in its midgut : either its fæces or its body comes to contaminate either food or water supplies in the house or locality. Swallowed by person in same house or locality. New host ingests L.D., (probably in flagellate form), acquires k.a.

This pure hypothesis : but it is suggested as the personal result of five years of close attention to and study of the problem. Will discuss in full next lecture.

Which insect? Only five worth considering: bed bug, sand fly, fleas, Conorhinus (which in nymphal stage like a bed bug), and possibly ticks or Stegomyia.

Practical Notes.

L. donovani and other haemoflagellates from culture are often very hard to stain ; as they wash off the slide very easily.

Shortt, H.E., Ind. Jl. Med. Res. April 1923. Vol. X. No. 4. p. 1166, recommends the following method, which gives excellent results.

1. Place a loopful of the fluid containing flagellates on a slide.
2. Add excess (4 loopfuls) of normal citrate saline and mix thoroughly (NaCl 0. 8 gm. Sod. citrate 1.5 gms. Distilled water 100 c.c.).
3. Spread out the liquid over an area of about ¾" square.
4. Fix for 15 seconds over vapour of 4 per cent. osmic acid.
5. Dry in the air as rapidly as possible.
6. Fix in methyl or absolute alcohol.
7. Wash in distilled water to remove salts.
8. Stain by Giemsa.

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LECTURE 7.

KALA AZAR (CONTINUED). CANINE LEISHMANIASIS, L. TROPICA, ORIENTAL SORE, ESPUNDIA.

Does bed bug transmit kala azar ? Indian variety is C. rotundatus vel C. hemiptera and not C. lectularius...Technique of bug feeds. Collect bugs. Keep in dry test tubes with dry filter paper and change paper often. Collect ova on paper. Dissect any bugs which die. Bed bug peculiar in having no natural protozoa of its own, as fully proved by Patton. Pure blood feeder also. Breed out from ova : dissect samples to ensure freedom from natural infections. Feed on healthy volunteer or shaved rabbit abdomen. Keep guaranteed stock going in lab. for work. Get k.a. case with numerous parasites in peripheral blood. Take him off treatment. Feed bugs on blood, (Patton).

Cornwall's bug feeding tube technique most useful. Container tube has in it bugs on filter paper, recently disturbed so as to feed readily. Shortt recommends a sp. gr. bottle for use as container tube. Feeding tube is smaller sized test tube : rounded end cut off, membrane of shaved young rabbit skin tied over flanged end under strict asepsis. Must be used fresh. Technique suggested by Knowles and used by Mrs. Adie at Shillong. Two operators : A does spleen puncture : B withdraws patient's blood into citrated saline from vein simultaneously. Mix blood and spleen juice in same syringe : put ⅓rd″ column in each feeding tube : insert in container tube, fix with plasticine so that bugs can crawl up filter paper and feed through rabbit skin membrane. This method first introduced by Sleeping Sickness Commission. Cornwall used it for feeding on mixed flagellate culture plus defibrinated blood. From 1915-22 used in India by several workers, (despite Patton's 1922 statement that he is the only one to use it in India). Very useful. Applicability to other insect borne diseases.

Four epochs and principal series of work on bed bug question :—1. Patton. 1907-12. Fed bugs on L.D. positive peripheral bloods. L.D. leaves degenerating leucocyte in bug's gut : becomes herpetomonad flagellate : 8th to 12th day bug's gut is swarming with flagellates and rosettes. Then flagellates pass down into hind gut and rectum. Lose flagella. Become small. Divide up. Post-flagellate stage like pre-flagellate L.D. form. N.B. true post-flagellate, has definite cell envelope, unlike Row's post-flagellate in culture. Post-flagellate forms found after 12th day from feed. Get into man either by crushing infected bug on abrasion on skin or via bug's fæces. But Patton's papers fail to prove the case. Note that at THIS stage Patton stated that a fresh feed on human blood washed out whole Leishmania infection in the bug.

2. Cornwall and colleagues, 1915-1922. Fed bugs as above on flagellate cultures plus defibrinated blood. After ingestion flagellates became enormous in size : visible under ⅔rds inch objective and high eyepiece : invested as if in sheath. Converted into writhing " thick tail " form with thick tail burrowing its way into mucosa of midgut. Then contract down to spherical motionless cyst, half embedded in gut wall. Cyst next ruptures and liberates second generation fine, thin, active motile, deeply staining flagellate : (cf. last lecture and B. M. Das Gupta's findings). Same process also found if fresh mucosa of healthy bed bug (but not other insect) mixed on slide with fresh flagellate culture. BUT, concludes Cornwall, this=reaction to either UNFAVOURABLE environment of bug gut : i.e. when nutrition exhausted flagellate goes into gut mucosal cell : or phase of attempted preservation. Concludes that bed bug is NOT the transmitting agent.

3. Recent 1920-22 work by Patton, Mrs. Adie, Cornwall and others. Mrs. Adie fed bugs with bug feeding tubes on mixtures of richly positive spleen juice plus patient's blood. Result=massive infection of gut : gut simply crammed full of L.D. flagellates, distended with flagellate culture. Patton's process of flagellation at once confirmed. Also saw thick tails and cysts as in Cornwall above : but only in dead dugs or in bugs with damaged mucosal membrane. Believes that :—(*a*) ordinarily L.D. flagellates in bed bug gut ; (*b*) if bug now again feeds infection is wiped out with exception of either (*c*) flagellates which ENTER CELLS OF MUCOSA of bug gut and there either become thick tails and finally liberate second generation flagellates into gut : or (*d*) flagellates enter into cells of mucosa and there unite by SYNGAMY, true sexual fusion, form zygote, inside which rosette of daughter flagellates form, are liberated on rupture of cell into midgut. These new daughter flagellates characterised by having macronucleus rather as scattered chromatin

dots than as single macronucleus. Also found mononucleate forms resembling Leishmania in salivary glands of bug from k.a. case's bedding, and Mackie 1922 has found the same. But these slides shewn to all expert workers in India who are agreed that they are NOT Leishmania. Christophers identifies them as a new species of Nosema, N. adiei. Mrs. Adie's findings very hard to interpret : plates very confused : statements vague. She thinks L. donovani may have cycle inside cells of mucosa of midgut of bug like that of Trypanosoma lewisi of rat in rat flea and mice.

Patton, 1922, considers her findings again confirm bed bug transmission. He has recently worked on bugs fed on flagellate cultures with feeding tubes. Finds that if N.N.N. cultures be taken from dissected bugs, (*a*) active flagellates are present in midgut of bed bug to 44th day after feeding and (*b*) hind gut gives positive cultures to 41st day. But Cornwall 1922 and Shortt 1923 repeated experiments, and have done extensive section cutting of infected bugs. Find no evidence whatever of intracellular cycle. Patton now considers case for bed bug absolutely proved : no further work necessary, only anti-bed bug measures. Cornwall considers case for bed bug absolutely negative : time to turn attention away from this much over-emphasised insect. He can find no evidence either that (*a*) viable forms are passed per rectum in bed bug ; (*b*) salivary glands ever infected ; (*c*) midgut ever serves as other than convenient situation in which flagellation occurs ; (*d*) no evidence for any intracellular phase. Says "let bed bug alone for a rest and turn to other possibilities." To sum up :— obvious that (*a*) bed bug's midgut in cold weather offers conditions similar to that of N.N.N. cultures at 22°C : and hence flagellate stage ensues : (*b*) also obvious that since bed bug has no natural protozoa of its own it cannot have been original evolutionary host of L. donovani, which must have been derived from some OTHER insect ; (*c*) real existence of any post-flagellate stage very much open to doubt. Why make problem so confused ? If L. donovani = insect herpetomonas, as seems almost certainly to be case, its extra-human cycle is probably very simple. It passes from pre-flagellate phase in human tissues into flagellate stage in some insect : ?? may be here a post flagellate stage which is reinjected into or ingested by man : and passes into fresh host as either flagellate or post-flagellate. Why postulate complex extra-human cycles ? Real, true extra-human cycle likely to be very simple. Mr. Hutchinson of Pusa now investigating truth or otherwise of intracellular cycle in bed bug : after three months work is still wondering as to best fixatives for infected bugs for section cutting.

Does bed bug transmit k.a. ? Impossible to say. But await some clear, definite, unquestionable piece of evidence before accepting. The k.a. transmission question really turns on extreme difficulty of infecting experimental laboratory animals. This the fundamental difficulty in all k.a. work. Mackie recommends flying fox as susceptible animal, Knowles not successful. Laveran advises white mouse, Italian workers young dogs. Macacus rhesus and sinicus monkeys both susceptible, and probably best. Injection of flagellate cultures very rarely succeeds. Row and Korke have infected monkeys with " Post-flagellate material." But very difficulty of infecting animals with flagellates suggests that flagellate is NOT the infective form to man. Knowles found that all sera, whether of k.a. or non k.a. persons, immobilise and finally destroy flagellate : this is not case however if serum decomplemented. Best mode of infecting animals is Laveran's MULTIPLE technique. Make thick emulsion of fresh p.m. spleen : inject everywhere : e.g. 20 c.c. intraperitoneal, 5 c.c. into liver, if male 2 c.c. into each testis, 1 c.c. by infiltration into each eyebrow. Or use spleen juice obtained by puncture and give intraperitoneally. Then one of five results follows :— (*a*) animal may never take at all and remain well and with liver puncture films and cultures negative. This most usual. 1922 animals in Calcutta shewed only so far four positives out of 82 experiments ; (*b*) after intradermal injection may get localised nodules as in Row's monkey ; (*c*) usual positive result is transient and symptomless leishmaniasis, (not k.a.). I. e. the animal remains well, shews no symptoms, peripheral blood examination and culture negative and also aldehyde test negative. But in 5 to 7 weeks if liver punctured may see few L.D. bodies and cultures on N.N.N. yield good flagellate growth. Puncture again a few weeks later and animal is now entirely negative. " Transient leishmaniasis." Or (*d*) may get true k.a. as in Knowles ' recent monkey. Have seen three such cases in monkeys : two at Shillong, M. rhesus, which both died literally of acute, fulminating k.a. with viscera full of L.D. after injection as above, and recent Calcutta monkey which died with fulminating k.a. six months after experimental feed. Hope it may now be possible to establish an animal strain and animal virus for experimental work. Difficulty of working with patients is that one must treat them, and then they become useless for experimental study and experiments. Mosquito transmission of malaria was finally proved by experiments on human volunteers. Personally consider that final and crucial experiments with k.a. will have to be carried out on man. Take batch of bed bugs, e.g. believed to be at infective stage. How the dickens can we test their infectivity when we have no susceptible animal other than man himself ??

Leishmania infantum, of Mediterranean k.a. probably identical with L. donovani. Especially affects children, (but of recent years in Assam children especially affected too). One point of difference if Mediterranean workers to be believed, animals much more readily infected with it, and both localised nodules and visceral infection result.

Basile insists that dog is the real reservoir of Mediterranean k.a. Found infected dogs in every k.a. house investigated in Bordonaro. Sergent brothers, Algiers, report interesting case : in isolated house outside Algiers they found child suffering from k.a., cat 4 months old, dog 2 years old, both animals also shewed Leishmania. Say 7% of dogs in Algiers shew L.D. In India all dog work so far negative (but cultural examinations not done).

Fact is that there exists a very definite disease of dogs, canine leishmaniasis. Two types : severe with k.a.-like symptoms : other chronic with paralytic symptoms. But canine leishmaniasis exists both in countries where k.a. and in others where no k.a. The canine parasite again exactly like L. donovani. Disease apparently spreads from dog to dog by dog flea, Ct. canis. Basile. Expt. 1, 1910. Reared puppies in lab. at Rome (where no k.a.). Took half to Bordonaro, where much k.a. Dogs here contracted leishmaniasis, died, on p.m. L.D. found. Controls at Rome were killed and examined. No parasites found. Expt. 2. Healthy dog taken, femur trephined, no L.D. found, proved free from disease. Its fleas collected. Let (a) of these=controls. Let (b) of the fleas fed on spleen of fatal case of canine leishmaniasis. Their midguts shewed flagellates : gut contents injected into a pup. Pup died 29th day, viscera full of leishmania. Control fleas and control dog negative. Expt. 3. Bitch and 2 puppies taken. Bone marrow of all 3 negative on examination. Fleas washed off with cresol. Now put into metal flea-proof cage with an old dog very sick with leishmaniasis. Fleas of sick dog attacked the three healthy dogs. Bitch never ill. One pup died 49th day, tissues full of leishmania. Second pup became ill, then recovered. Basile found dog fleas in bedding of all dog owners in Bordonaro. Expt. 4. Collected such fleas from Bordonaro , infected area, and took to Rome (free area). Fed them in Rome on dogs known by bone marrow examination to be free from disease. Dogs got leishmaniasis : control dogs' exposed to Rome but not to Bordonaro fleas remained well. Basile concludes that canine leishmaniasis is disease of dogs, due to a leishmania parasite, and is transmitted from dog to dog by dog flea and that this parasite=Herpetomonas ctenocephalis. So far he appears to be right. Next, says he, sometimes these infected dog fleas bite children instead=Mediterranean kala azar: and thus L. infantum=H. ctenocephalis. Of 1000 wild dog fleas 4 shewed flagellates. But is H. ctenocephalis really same as either of these two parasites, or is it natural flagellate of flea ?? Of 200 dog fleas collected by Noller in Berlin, where there is no canine leishmaniasis as far as is known, 24 had herpetomonads. Fantham found 3 out of 68 dog fleas in England infected, no dog disease. Basile concludes flea is transmitting agent of k.a. and infection passes via its fæces or via scratching the crushed flea into an abrasion.

Basile's lead followed by others. Laveran and Franchini, 1913, produced leishmania infection of rats and mice with herpetomonad of dog flea and with that of sheep ked. Fantham and Porter, 1914-16, did similar experiments with dogs, mice, birds, fish, reptiles. Herpetomonads of house fly, sand fly and even of Euphorbia latex produced leishmaniasis. Problem becoming a very muddled one. Statements made by these authors not very convincing. Claims seem exaggerated. Also what earthly connection can there be in nature between such a herpetomonad as that of the sheep ked and a white mouse, or flagellate of the water beetle and a white rat ? " Leishmaniasis in the making " : but is it so truly ?? In 1921 most careful, full, thoroughly scientific bit of work by Hoare on same lines, working with herpetomonad of sheep ked. Was not only quite unable to confirm any one of above claims but hundreds of careful experiments quite negative. Here note : it seems to me that these workers are all at the wrong end of the stick. To start off with insect herpetomonad of almost any insect and try to produce kala azar with it in animals will not lead us much further. We have got to start with k.a. of man, and pucca L. donovani, and then go to the insect or other agent. Basile appears to have proved that canine leishmaniasis may be due to H. ctenocephali, but nothing more. Any connection with human k.a remains unproved. Patton's classical dog case, dog experimentally infected with k.a. developed piroplasmosis : peripheral blood absolutely crammed with **BOTH** piroplasma and leishmania. Thousands of Ct. felis fleas collected and fed on dog. Ingested parasites : but after 6 hours not a trace of a parasite in the fleas. Simply degenerated. D'Silva 1913, and Wenyon, 1914 flea experiments in Mediterranean k.a. all negative. Franchini thought anopheles transmitted but at once shewn by Patton that he was working with anopheles with natural herpetomonad infection of their own.

Case for Conorhinus. In its early nymphal stages it has the habits of a bed bug. Found by Awati in Assam 1922, in all k.a. areas but not found in k.a.-free area of Jorhat. Occurs equally in Assam jungle village and Calcutta city. Shortt at Shillong has, I understand, produced L.D. flagellates by feeding Conorhinus on k.a. spleen juice. Experiments at present going on in Dr. Strickland's laboratory, so far no results positive.

Case for sandflies. Occur in both Assam jungle and in Calcutta. First sandfly discovered was in Calcutta. Mackie found 10% of sandflies in Assam infected with a natural herpetomonad of their own. Again ? is this a parasite of the sandfly only or is it=L. donovani ?

Napier has recently analysed his Calcutta cases. (*a*) Of about 200 kala azar cases who have come from outside into Calcutta for treatment most live in northern half of city. These cases dotted about all over Calcutta. (*b*) Of over 200 cases of k.a. in permanent residents in Calcutta city considerably more than half occurred in persons residing in a small area near Marquis Street and just behind the Wellesley Street tank. This area appears to be the home and endemic focus of k.a. in Calcutta city. Anglo-Indians in this area are affected in far greater prevalence than their proportion to the general population of the area. This focus should be at once investigated, as important results may follow. The houses are mostly old stone ones, damp, with much adjacent vegetation and obviously exactly suitable for sandfly breeding. There is less over crowding and fewer bed bugs here than in kala azar-free northern Calcutta.

Transmission problem, as will be understood, very complex, and becoming very confused. Consider it essential at this stage to stick to (*a*) simple known facts, (*b*) try to get collateral evidence from the known epidemiology, (*c*) work ONLY with clean, laboratory bred insects Only four appear worth considering :—(*a*) bed bug, where only thing to do now is section cutting to confirm or disprove cycle, and experiment on human volunteers ; (*b*) sandflies ; (*c*) Conorhinus in its larval stages ; (*d*) fleas.

Having decided as working hypothesis (only) that the parasite gets out of the body via peripheral blood, suggest let us now study the other end of extra-human cycle, study modes by which natural infection could occur, such as oral and subcutaneous : and study under what conditions the flagellate may pass back into L.D. form. Here recent results of some interest. Flagellate will survive in frog's stomach up to 3 days despite sepsis. If injected into frog's anterior lymph sac flagellates pass through the body and intracellular L.D. bodies as well as free flagellates seen in spleen and liver. Infection however killed off in a few hours. Experimental work begun to study behaviour of flagellates with gastric juice, whole blood, lymph, etc.. behaviour at different pH ranges and different temperatures and wet bulb states. If we can get clear information of the OTHER end of the cycle we may get at something.

Also collateral evidence. Napier's k.a. map of India : progress of disease arrested at 1500 feet, distribution almost e. g. follows that of rainfall from the Bay of Bengal monsoon. McCombie Young's recent work, analysis of time of year of onset of 5000 Assam cases. In every infected district smallest incidence in September: in all a marked rise in November. But as one passes down Assam from acute k.a. areas such as Sibsagar to sporadic areas such as Sylhet big peak of incidence changes from January to February, March etc., till it is in July in Sylhet. Does this correspond with distribution of any biting insect ??

Incidentally so much already known that if some clear headed worker would only try to tabulate and study it all he might be able to suggest a further advance.

Leishmania Tropica.

Leishmania tropica, first discovered in Boston, U.S.A., by J. H. Wright, 1903, in oriental sore of an imported Armenian child. Disease known since 1756 or earlier. Nicolle first shewed that L. tropica passes into flagellate stage on N.N.N. Parasite found in sores and in blood films taken from just near the margin of the sore. To see clean the sore up with swab soaked in spirit and let it dry. Get clear, serous exudate, not pus and not blood : either by capillary pipette, or puncture growing edge of sore, let blood escape, clot, then get clear serum that comes afterwards. Parasites found :—1. free in masses between cells ; 2. in immense numbers in endothelial cells, up to 50 or more per cell ; 3. in polymorphonuclears where being phagocytosed, and 4. in large hyaline mononuclears.

Sore itself characterised by 1. ulceration (not always, but always skin atrophy) ; 2. cellular infiltration of corium by endothelial and wandering plasma cells packed with parasites ; 3. inflammatory reaction, formation of granuloma tissue. Easily mistaken in some rare cases where it occurs not on skin but on mucous membranes for hard chancre : Kasauli case on lip of British officer mistaken for syphilis.

Parasite as cause fully proved. Virus inoculable. Experimental inoculations by Wenyon and others into themselves into abrasions, bullæ etc. After incubation of about 16 to 24 days FEVER sets in, transient (?? is this accompanied by a transient visceral leishmaniasis ??) : then fever disappears and typical sore full of parasites results. Jews of Bagdad inoculate female children on buttocks to preserve them from sores on face etc. in adult life and disfigurement. Bouilliez, 1917 accidentally inoculated himself into eye when dissecting infected mouse : lesion on conjunctiva.

Cultures. In N.N.N. parasite assumes flagellate form just like that of L. donovani. Whole morphology of L. donovani and tropica similar. Only tropica able to withstand a good deal of sepsis. Flagellates up to 25 μ long, first appear in culture about 4th day. Row considers that tropica grows at slightly higher temperatures than does donovani, and describes minor differences in average size etc. of two species of flagellates.

L. tropica infection much more easily established in animals than donovani. Nicolle and Sigre, 1908 first infected monkey with nodule. Nicolle and Manceaux, 1910, proved dogs very susceptible. Wild dogs found infected in nature : Neligan, 1913, found leishmania in sores of dogs in Teheran. Gaucher found 15 out of 21 random dogs in Teheran had sores showing L. tropica. Other records from dogs in Tashkent, etc.

Dogs, monkeys, white mice especially susceptible. Gonder, 1913, shewed that if L. tropica injected into white mouse intraperitoneally or intravenously generalised leishmaniasis of the viscera followed. Much classical work by Laveran, (*a*) for localised lesions : tiny flap cut into eyebrow, insert bit of nodule under skin. Or infiltrate skin with needle, intradermal not subcutaneous injection. Sore follows in from 20 to 60 days. Even flagellate cultures may take this way. At moment of appearance of sore animal very susceptible, may inoculate himself elsewhere and get secondary nodules. Again Row considers post and super-post flagellate phase of culture most infective. (*b*) Laveran using technique with vaccination needle got experimental sores in animals in 8 days. Sores last 8 months in animals. Some laboratories have kept up experimental strains for years. Best mode of all is to inject emulsion of L. tropica into testis of white mouse : testis becomes gangrenous : L. tropica generalises throughout viscera, kills white mouse, spleen enlarged, and testes and viscera packed with leishmania. Local sores produced on scarified rabit cornea.

Crossed immunity experiments of Laveran. 1. Got dogs. Repeatedly inoculated them with L. tropica, sores followed, until animals so immune that they failed to take again : now immune to L. tropica. Also monkeys ditto. Three months later injected with 8 c.c. spleen emulsion from k.a. case. Monkey died on 55th day, lost 370 gms. weight, in films no L.D. seen but N.N.N. cultures of spleen richly positive. Thus monkey immune to L. tropica still susceptible to L. donovani. 2. Conversely three monkeys inoculated into liver with L.D. One died, tissues full of L.D. Others no symptoms ?? immune or ?? not susceptible. These two now inoculated three months later with L. tropica. Developed oriental sores full of L. tropica.

Transmission problems. Note first distribution of oriental sores is on exposed skin areas : thus not usual on knees, but common in Mesopotamia on bare knees of Tommies wearing shorts. Patton again goes for bed bug. Bugs fed on blood near edge of sore containing parasites shew L. tropica flagellates. Almost impossible that bed bug is transmitting agent : oriental sore found in Mesopotamia desert camps where bed bugs all killed out by the heat and little or no harbourage for them. Fact is, as now proved, that T. lewisi of the rat, L. donovani and L. tropica of man, H. culicis of mosquito, H. muscæ domesticæ and H. ctenocephalis of dog flea will all live in motile state in bed bug's midgut for 44 days or even longer : bed bug's stomach provides essential conditions of sterility, HB., etc. just like N.N.N. culture : but this no proof that bed bug has anything to do with any of these parasites. Again Patton thinks he has seen an intracellular phase, as with L.D. Cornwall fails to confirm him. Whole weight of evidence against bed bug.

Case for house fly. Blanc and Caminopetres think disease handed on by direct contact. Flies fed on sores, later contents of gut inoculated into abrasions, but NO sore followed. Laveran says curves of oriental sore incidence and fly infestation parallel. Former in Algiers follows latter and parallel two months later. Suggests fly feeds on sore, then goes to healthy person and infects abrasion. Patton found that L. tropica degenerates in fly's gut, but may fly not carry mechanically on pads of feet ? Wenyon favours this view.

Case for sandfly. Sergent brothers 1921 collected 94 sandflies at Biskra (hot bed of the disease), posted to Algiers where none. Only 7 survived. Emulsified in saline, inoculated into abrasion on skin of arm of person never near any oriental sore case. Lesion healed. 2 months and 24 days later oriental sore developed at site, full of L. tropica. Acton shewed that distribution on surface of body of (*a*) oriental sores and (*b*) sandfly bites are identical. Laveran and Franchini produced both oriental sores and visceral leishmaniasis in mice and guinea pigs with sandfly herpetomonads. May now take it, 1922. as proved that sandfly is transmitting agent of oriental sore : but exact steps of transmission have still to be worked out. If so does L. tropica=natural herpetomonad of sandfly or not ???. Further problem : sandflies, in absence of man, feed voraciously on lizards. Is gecko cold blooded reservoir of L. tropica ?

Case for direct inoculation from man to man. At Biskra, a hot bed of the disease, at certain seasons of the year almost every sore among the French troops of any septic origin becomes a true oriental sore. Case of Marie Gros : came to Biskra from Marseilles, where no oriental sore. Lived with sister who had numerous oriental sores. Used same towels etc. Two months later developed oriental sores on face and forearms.

To sum up sandfly probably certain carrier, and almost proved. Gecko possible reservoir. Direct inoculation not unlikely. This lends weight to case for sandfly in kala azar. But, on other hand, very marked differences between the two diseases may suggest sandfly not the carrier in k.a.

Fourth type of human leishmania infection is Espundia, or S. American leishmaniasis. Most interesting disease. Usually affects mucous membranes, especially of angle of mouth and naso-pharynx. But may affect only skin : note that oriental sore is almost always skin lesion but may affect mucous membranes, espundia is converse. Known since days of ancient Incas. Parasite, which is practically L. tropica, discovered by several Brazil workers almost simultaneously in 1909-10. Problems of espundia in Brazil similar to those of oriental sore in Europe and Asia. Dogs again, of course, suspected. Pedruso found infected dog with nasal ulcers shewing parasite living in house of case with espundia. Cases of the disease among woodcutters who have been months in the jungles away from all human habitations. Townsend, 1915, suspected Chironomidæ as transmitters. Found herpetomonads in their gut : produced local papule in guinea pig by scarifying and rubbing in fly gut contents. Phlebotomus cruzi of S. America regarded as probable vector. Cerqueira, 1920, records case of professor who had typical espundia lesion at site of bite of this insect, and refers to four other similar cases.

Note :—" Histoplasma capsulatum " described as a leishmania-like parasite by Darling in Panama, 1907, in spleen of Chinaman is a yeast, and now named Cryptococcus capsulatus. Wenyon, after examining these workers' own materials states that " Plasmosoma jerichœnse " of Jericho boil, Huntemuller, 1914 : and " Toxoplasma pyrogenes " of Castellani, 1913 in tropical splenomegaly are respectively degenerated or badly fixed and stained L. tropica and L. donovani. These three parasites are histories of mistakes.

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LECTURE 8.

THE TRYPANOSOMES.

Leishmania lives in cells, either endothelial or leucocytes : malaria parasites live in R. B. Cs., Trypanosomes however live free in plasma and are especially parasites of the lymphatic system. Innumerable species, and infect fish, amphibians, reptiles—(in which case are transmitted by leeches) : birds, animals and man, (in which case are transmitted by biting insects). Structure of trypanosome : cell membrane definite but elastic and compressible, posterior micronucleus deeply staining, dot or rod or bar : central macronucleus, less well staining : basal granule, flagellum arises just in front of micronucleus from basal granule : passes forwards along cell body, raising cell membrane and included cytoplasm as it does so into an undulating membrane, and finally projects free. Move with micronucleus behind : and free flagellum in front : like a snake wriggling backwards. Presence or absence of food and volutin granules in cytoplasm useful point in differentiation of species. Under cell membrane lie delicate, contractile threads of protoplasm, running from pole to pole like lines of longitude around earth=myonemes. When these contract trypanosome becomes short and stumpy, when they relax becomes long and thin. Well seen in T. rotatorium of frog. Dispute as to true polarity of trypanosome. Trypanosomes can easily reverse and wriggle backwards : also similarly for Herpetomonas.

Shapes of trypanosomes vary enormously. T. rotatorium of frog 60 μ long, 28 broad, fat and motility sluggish. T. vivax of cattle 28 μ long, 1½ μ broad, moves with almost incredible rapidity across the microscope field, scattering the R. B. Cs. like a minnature cyclone. Roughly trypanosomes in general may be classified as follows :—

1. Trypanosomes of cold blooded animals and fish. Examples T. rajae of skate, T. rotatorium of frog, T. vittatæ of turtles. Transmitted by leeches.

2. Small group hitherto only known in gut of biting flies : and which are almost certainly here merely developmental

stages of mammalian trypanosomes whose mammalian hosts have not yet been discovered. E.g. T. grayi of Glossina palpalis.

3. Trypanosomes of birds, e.g. T. noctuæ of little owl, the subject of Schaudinn's classical muddle (vide later).

4. Trypanosomes of mammals; (*a*) pathogenic and (*b*) non-pathogenic. Examples innumerable: e.g. T. duttoni of mice, T. cuniculi of rabbits, T. indica of Indian ground squirrel, trypanosomes of monkeys, elephants, carnivora. Will however only consider the commonest.

1. *T. lewisi* of rat. Best example as full life cycle known. Discovered by Lewis, 1878, in Calcutta rats. Found in from 20 to 50% of rats the world over. (Appears to be seasonal in incidence in Calcutta. Also batches of rats from different areas may shew all infected or none infected). Rats usually shew no symptoms even if their blood be alive with trypanosomes. Very slender, pointed and highly active motility. 25 μ long by 1½ broad. Macronucleus at junction of anterior and middle thirds of body. Micronucleus rod like.

Rat gets infected from either scratching in infected faeces of rat flea or rat louse, or by swallowing infected insect. Incubation 6 to 7 days. Trypanosomes appear in peripheral blood 8th day onwards. At first shew immense variety of shapes, size and form = phase of invasion = characterised by pleomorphism of forms in blood. Normal forms, small stumpy forms, dividing forms, long tailed forms, rosettes of actively dividing forms, (like rosettes of flagellate L.D. but here the trypanosomes all have their flagella pointing away from centre of rosette). Multiply by longitudinal binary fission. Period of invasion characterised by immense variation of forms in blood films. Blood then continues for months or years to shew trypanosomes from time to time, intermittently. N.N.N. blood cultures often positive when films negative. By degrees pleomorphism ceases, and when infection well established most forms seen are constant to type.

During periods of absence from peripheral blood the trypanosomes are away in internal organs: schizogony multiplication cycle in internal viscera keeps up supply to blood and lymph. Trypanosomes in viscera pass into endothelial cells. Micronucleus divides into two: half disintegrates: second gives off chromatin granule which passes to and ?? fuses with macronucleus. Flagellum lost and remaining nuclear matter disappears. Chromatin divides into 2, 4, 8 etc. Finally in cell a rosette of young Leishmania-like forms. Then flagella grow again; cell ruptures, young small trypanosomes set free from rosette : either re-enter endothelial cells or pass into peripheral blood where they become adult in type.

Rat cycle thus consists of (*a*) invasion period with schizogony in peripheral blood, and (*b*) multiplication through a leishmania phase in viscera with the production of broods of trypanosomes which pass to peripheral blood at intermittent intervals.

Infection transmitted by both rat flea and rat louse. Several possibilities. 1. Flea feeds half meal on infected rat : completes meal on healthy rat : trypanosomes carried mechanically from one rat to other on flea's proboscis. 2. A few hours later proboscis now not infective : even contents of insect gut not infective. True insect cycle has started. In this connection in trypanosomiasis we may have either (*a*) development only in insect proboscis, e.g. T. vivax of catttle in tsetse fly proboscis : (*b*) development only in anterior gut, as in fish trypanosomes in leeches, where trypanosomes multiply in leech crop : (*c*) development in whole length of insect or leech gut : e.g. T. rajæ of skate. Trypanosomes multiply in leech's midgut : non-motile leishmania forms pass into rectum : develop flagella, pass again forwards to proboscis, thus infect fresh fish ; (*d*) development in insect gut but infective forms pass to and collect in salivary glands, as in T. gambiense, vide later ; (*e*) develop in gut but infective forms passed in fæces This last is the case with T. lewisi in rat flea. Trypanosomes pass into flea midgut. Enter cells of midgut mucosa. Lose flagella, become rounded, form intracellular pseudocyst. Inside cyst divide to form cluster of small daughter trypanosomes. Cell ruptures, daughter trypanosomes set free, pass down into rectum of rat flea or rat louse (cycle in both the same). By repeated division and multiplication small forms produced in myriads in rectum : these lose flagella, become leishmania-like, passed in fæces. Flea or louse's fæces infective if scratched into abrasion of healthy rat : also insect infective if eaten by rat or its body crushed into abrasion in scratching.

Finally (*f*) possibility considered that trypanosome infections in biting fleas may pass to ova and next fly generation ?? In T. lewisi rat flea's fæces become infective on 7th day after feed, remain so for 45 days or—probably—much longer.

2. *T. evansi*, the cause of surra, in camels, horses, mules, elephants, often very prevalent in Indian cavalry regiments. Trypanosomes found usually in myriads in drop of fresh blood. 22 to 30 μ long, 1 to 2 broad. Discovered by Evans, Punjab, 1880. World wide distribution. This trypanosome is mono-

morphic, little variation in size and shape. Believed that transmission carried by biting stable flies of Stomoxys, Tabanidæ etc. group, but nothing proved or worked out. Tabanus striatus infective via the proboscis, mechanical transmission, up to 15 minutes after feed. Also carried from horse to horse by horse bites. T. mbori, annamense, soudanense, elephantis, berberum, marocanum, hippicum and venezuelense are all probably T. evansi.

3. *T. brucei* causes nagana disease in animals in S. Africa. Fever and paralysis. Plimmer and Bradford, 1899. 28 to 30 μ by 1½ to 2½ μ. Rather blunt anterior pole, granules in posterior half. Very pleomorphic. Produces fatal disease in all domesticated animals, e.g. oxen, and is very pathogenic to laboratory animals. Probably identical are T. pecaudi, togolense, ugandæ, equi. Transmission from animal to animal via tsetse flies, also possibly by biting Tabanidæ etc.

Is this parasite the same as T. rhodesiense or not ? Vide later.

4. *T. equiperdum*, cause of dourine, venereal disease of horses, mules, donkeys, etc., transmitted by coitus. Acute inflammatory œdema of sheath of penis or vagina, anæmia, wasting, skin eruptions, paraplegias. Like T. brucei but has no granules. Very monomorphic. 16 to 34 μ long. Trypanosomes found in very scanty numbers in blood, (15 to 20 c.c. of blood may be infective), and cerebro-spinal fluid : but swarm in the œdematous fluid of genitalia. Role of biting insects here not important.

5. *T. equinum*. Cause of mal-de-caderas of S. American horses. Monomorphic and slender. Micronucleus very minute. Causes acute disease in laboratory animals. Symptoms progressive paralysis, hæmaturia, wasting, albuminuria. Infection in horse usually fatal. Probably transmitted by Tabanidæ flies, but nothing proved. Rodents believed to be reservoir of infection, e.g. the capybora of S. America.

6. *T. theileri*. Huge trypanosome of cattle in Transvaal, India, Japan, 60 μ long, 5 μ broad. Once believed to cause biliary sickness of cattle but this now known to be due to Piroplasma. Apparently non-pathogenic.

Any number of other animal trypanosomes : many possibly identical with one or other of above six. Pass to the two great human trypanosomes, T. gambiense, the parasite of C. African sleeping sickness, and T. rhodesiense, cause of S. C. African sleeping sickness.

7. *Trypanosoma gambiense.* Immense range of this parasite : covers three quarters of Africa. Senegal to Angora, whole Congo basin, Uganda, south as far as Rhodesia, whole of E. Africa, north to Sudan fringes, west to Timbuctoo and Nigeria. Possibility of introduction into India from E. Africa of sleeping sickness. Found in African islands, Principe.

Castellani's extraordinary views with regard to "Castellanella castellanii." Parasite first discovered by Ford (material studied by Dutton), in Gambia 1902 : re-discovered by Castellani in Congo, in 1903 and by him still called C. castellanii.

Methods of examination of man (or animals) for trypanosome infections :—

1. Fresh drop of blood under microscope, or dark ground every time. 2. Stained thin or thick blood films. 3. Skin papules or local rashes, lesions etc. 4. Take 10 to 20 c.c. blood into citrate saline, centrifuge, examine deposit. Third deposit often shews scanty trypanosomes. 5. Examine cerebro-spinal fluid, and centrifuge if necessary. 6. Puncture fluid from enlarged lymph glands. 7. Examine laboratory animals to make certain they are free from natural trypanosomes. Inoculate with 10 to 20 c.c. of suspected blood. N.B. Laboratory animal used must be susceptible to trypanosome in question. 8. Blood may shew auto-agglutination of R. B. Cs., phenomenon very characteristic of trypanosome infections. In fresh blood drops instead of rouleaux we get irregular, agglomerated clumps of R. B. Cs. 9. Try complement deviation on lines of Wassermann but with antigen prepared from trypanosomes freed from R. B. Cs. by washing. 10. Culture of blood or lymph on N.N.N. Animal trypanosomes grow better than human. Negative findings in 1 to 10 have no value at all.

T. gambiense. In peripheral blood very pleomorphic. Short stumpy forms, 14 to 20 μ long, slender forms, 23 to 33 μ : intermediate forms. Eosinophile vacuole in front of micronucleus often stains well. Forms in cerebro-spinal fluid, multinucleate, polyflagellate, and leishmania-like. Mode of multiplication studied after inoculation into laboratory animals. When T. gambiense inoculated thus, infection occurs only occasionally, and runs a chronic course in animal. Multiply in animal blood by longitudinal binary fission. Just like T. lewisi. Initial phase with pleomorphic forms in blood : then peripheral blood clears and multiplication with leishmania like phase in internal viscera. Breinl and Moore describe forms where micronucleus and flagellum discarded ?? Visceral cycle just like that of T. lewisi of rat. T. gambiense cultivated with some difficulty on N.N.N. Cultures viable for 37 to 45 days but does true multiplication take place ?? Crithidial and leishmania forms met with in N.N.N. cultures.

Here note certain serological phenomena in connection with trypanosome infections. If serum of immunised animal be added to culture of specific trypanosome concerned, agglomeration of the trypanosomes occurs : almost like Widal test. Trypanosomes agglomerate into primary clumps, and these again into secondary clumps. Also note in all trypanosome infections the " trypanolytic phase." Is a crisis preceded by " granule shedding." Just before trypanosomes disappear from peripheral blood they start (*a*) to shed granules. Granules in a trypanosome of two characters, volutin granules and shed granules. Latter appear to form from macronucleus, pass down flagellum, and are cast off. Stated that they are capable of then again growing into new trypanosomes ?? After granule shedding (*b*) trypanolytic crisis and trypanosomes disappear from peripheral blood, but at same time exacerbation of symptoms from ? liberated toxin. ? Phases of T. gambiense infection in man correspond to those of T. lewisi in rat. 1. Period of first invasion of peripheral blood with pleomorphic forms. 2. Invasion of central nervous system fluid with crithidial, leishmania and aberrant forms. 3. Multiplication phase in internal organs, blood free. 4. Reinvasion of peripheral blood with less pleomorphic forms. 5. Granule shedding, trypanolytic crisis, then again disappear from blood etc. Cyclic invasion of blood.

Transmitting agent of T. gambiense is the tsetse fly, Glossina palpalis. In Uganada 3 to 4 per 1000 wild flies infected. In Nyassaland 13 per 1000 wild flies infected with trypanosomes of the brucei group. (Term brucei group is often used for T. gambiense, rhodesiense, brucei, evansi, lewisi, etc. with general size and morphology very like brucei). In experiments 5 to 6 % of flies can be infected in the laboratory, or even 21 % if previously starved. G. palpalis lives close to water's edge : hence peculiar distribution of epidemic disease, along shores of great lakes and edges of great rivers. Note anatomy of Glossina : gut = oesophagus, proventriculus, thoracic gut, abdominal or midgut, hind gut. Coiled long salivary glands lie in abdomen : ducts pass forwards through thorax and open on hypopharynx. Fly identified when at rest by crossed wings, like a pair of scissors. Possibilities are :—1. mechanical transmission ; half feed on infected case, completes feed on healthy person, mechanical transmission on proboscis. This mode possible only for first two hours after feed. 2. Then trypanosomes pass down to midgut, and here at this stage are NOT infective, even if collected and injected into laboratory animal. Why do only some 5% take infection?? Note that a second feed of blood will clear infection in fly often. On other hand in some flies infection persists for whole life of fly. Development of trypanosomes now occurs and fly is not infective again to man until 20th day after feed. Trypanosomes especially the short, stumpy ones, accumulate in posterior part of midgut : by binary fission multiply till, about 8th day, gut is simply crammed with trypanosomes of all sorts and sizes and shapes. By degrees long, slender forms collect and begin to pass forwards again to midgut and proventriculus. Forms in posterior part are reservoir from which these slender forms are kept supplied to proventriculus. The latter pass on to hypopharynx, thence back down the salivary ducts to salivary glands. Attach themselves to wall of gland acini by flagellum : become broader, assume crithidial type. Divide rapidly till fly's glands become packed with infection. Fly never infected till salivary glands are invaded. When such fly feeds again it injects the small trypanosomes into man, who thus acquires infection. Incubation period in laboratory animals on which infective flies fed=2 to 3 weeks. No viable forms passed in fly's fæces : gut contents experimentally also infective, but not until after the period of invasion of the salivary glands.

In addition to fly transmission some evidence that disease may spread by coitus. Koch's 15 cases in women in Kisba, where no tsetse, but whose husbands all infected. Infected rats do not transmit by coitus : but Hindle found that if a drop of infected blood be introduced into vagina infection took, (six positives) : and even got infection by putting drop of very virulent blood on unbroken skin. Question also raised as to possible hereditary transmission to human offspring, but nothing proved. Both T. gambiense and T. rhodesiense may be found in milk of infected mothers. Fishing is the one great occupation especially connected with exposure to infection.

8. *Trypanosoma rhodesiense*, parasite of sleeping sickness of South Central Africa. This form of trypanosomiasis is much more sporadic and rarely epidemic : also has no special association with water edges. T. rhodesiense very similar to T. gambiense but with one important difference. Inject both into rats. In the case of T. rhodesiense a certain proportion of the trypanosomes change, 5 to 6% : the micronucleus moves forwards till it becomes anterior to the macronucleus :

" posterior nucleate forms ", and this does not happen with T. gambiense.

Here note the difficulty often experienced in deciding as to what species of trypanosome is present when dealing with any member of the very similar brucei group. Several methods open. 1. Take very careful measurements of about 1000 individuals : e.g. measure total length : distance between micro- and macronuclei : distance between micronucleus and posterior pole.

2. Different susceptibility of laboratory animals to different strains. Thus T. vivax not very fatal to laboratory animals but occasional rabbit dies in 5 to 12 days. T. brucei very fatal to most laboratory animals. T. rhodesiense much less fatal. With T. gambiense difficult to get animal to take at all. 3. Different transmitting flies. Feed different Glossina species on infected animals, and find which transmits : this will often identify a difficult trypanosome. 4. Immunity reactions. Immunise animal with trypanosome A : its serum becomes immune and causes in vivo and in vitro agglomeration and finally destruction of trypanosome A. But this serum has no action on trypanosome B. Animal immune to T. gambiense (where laboratory infection is usually a chronic disease and then recovery) still quite susceptible to T. rhodesiense : and vice versa. Clearly different species, as established by this and by different vectors etc. N.B. Trypanosomes can be cultivated on N.N.N., but cultures of both gambiense and rhodesiense, if so cultured, soon lose infectivity to animals.

Cardinal question about T. rhodesiense is " Is it or is it not the same trypanosome as T. brucei, the cause of nagana in wild game of South Central Africa " : and does wild game act as reservoir for infection ? Blacklock and Warrington Yorke of Liverpool School insist that the two are identical. Evidence : Glossina found in districts cleared for four years of human inhabitants still found infective to monkeys. Duke's two fly boys, 1915, resided 18 and 33 months respectively on uninhabited lake shore, cleared of human population five years previously : went down with sleeping sickness. Widely scattered cases in North Rhodesia favour idea of an extra-human reservoir. Rhodesian disease rarely, if ever epidemic. Week, 1914, infected an antelope with T. rhodesiense from human case: its blood was infective for five weeks, though animal never became ill. Cows found in natural state infected with T. rhodesiense (observations doubtful). These workers hold T. rhodesiense=T. brucei : game is reservoir and principal focus of disease : man only accidentally infected, and cases sporadic.

To exterminate the disease it will be necessary to exterminate all wild game and place all domestic cattle under observation.

This view strongly opposed by others. Dr. Ascensio and two Africans injected with blood from infected cow : did not take : species of trypanosome most unfortunately not stated, (may have been vivax ??). Taute's big series of experiments. 1. 1918, fed upon himself laboratory bred G. morsitans infected with T. brucei from wild game. Nothing doing. 2. Took a dog infected with same trypanosome : injected 2 c.c. of its blood into himself: again nothing doing. Trypanosomes simply died out in his blood. 14 days later he took 90 c.c. of his own blood and injected it into laboratory animals susceptible to T. brucei infections : none took. 3. Took blood from 4 horses and 2 mules with natural T. brucei infections : injected himself, Huber, and 129 human volunteers. None took. At same time however injected as controls susceptible rats, dogs, and a goat, all of which took.

Yorke and Blacklock explain away these experiments by saying man is very resistant to trypanosome infections in general and to T. rhodesiense=T. brucei in particular : but this seems rather begging the question.

Matter still at issue : but so far evidence is in favour of view that T. rhodesiense is NOT T. brucei. Note that whereas with the bacteria (e.g. B. pestis) and spirochætes (which are almost certainly NOT protozoa) there may be animal reservoirs of diseases which attack man : yet the protozoa of man are curiously specific and it is difficult to infect animals even experimentally. "Reservoirs" a most convenient theory but in protozoology at least, one that should be accepted with very great reserve.

T. rhodesiense infection transmitted by Glossina morsitans : fly distribution and hence disease distribution therefore different to that for T. gambiense. Fly cycle very similar to that for gambiense. No mechanical transmission after 24 hours. Fly becomes infective after 14th day. Fly cycle remarkably affected by temperature: experimental flies not infective, although trypanosomes still in gut, at 55 to 65°F : only become infective at 75 to 80°F : hence immunity of higher altitudes from the disease. 2.2 per mille of wild flies shewed T. rhodesiense in Luangwa valley : 78 miles away on the Congo Zambesi watershed where temperature lower with altitude only 0.7 per mille found infected.

In addition to sleeping sickness :—1. Professor Lanfranchi accidentally infected in Paris laboratory with T. evansi : distinguished professor's papers clearly shew he has not contracted sleeping sickness. Trypanosomes simply disappeared after few days. 2. Macfie records case of man infected with T. vivax of cattle without symptoms on Gambia coast.

Those interested in kala azar problem would do well to study trypanosomiasis literature : it is full of suggestive, possibly analogous points, and information.

3. *Schizotrypanum Cruzi.* Parasite of special interest to Indian kala azar workers : seems exactly intermediate between parasites of African sleeping sickness and Indian kala azar as cause of disease. S. cruzi better considered separate genus, as is a trypanosome with such peculiarities. Cause of Chagas' disease or South American trypanosomiasis. Parasite first discovered by Chagas in 1909 in midgut of transmitting reduviid bug, Triatoma megista or Conorhinus megistus : later discovered in spleens of infected children. Symptoms of disease : fever, enlarged lymph glands, big spleen and thyroid, myxœdematous swellings, endocrine failure, sometimes or often meningo-encephalitis (? are there ever any meningeal symptoms in kala azar or invasion of the central nervous system ?).

This curious parasite perhaps best studied from injection of parasite into man. Bug injects a crithidial type of trypanosome which passes to internal organs, especially spleen, liver, bone marrow, thyroid, testes or ovaries, but makes—above all else—for two sites : viz. the striated muscle system, e.g. muscles of the skeleton, heart, legs : and central nervous system neuroglia. Here parasites enter into (*a*) muscle cells, (*b*) neuroglia cells. Lose flagellum and come to rest as leishmania forms, 2 to 4 μ in diameter. Next divide by repeated binary fission until infected cell becomes a pseudo-cyst crammed with leishmania forms. According to Mayer and de Rocha-Lima the leishmania forms are of two types, large round forms which predominate and multiply rapidly, and small round forms which are about to become trypanosomes.

Striped muscle fibre or neuroglia cell become a sac distended with leishmania forms. Bursts. Parasites liberated and either (*a*) enter adjacent cells to repeat cycle : or (*b*) pass into peripheral circulation where they become trypanosomes. After 10 to 12 days peripheral blood shews trypanosomes derived from this endogenous cycle. Any forms developed as result of this cycle develop in peripheral blood into typical adult trypanosomes free in blood plasma.

According to Chagas, however, schizogony cycle of endothelial cells invaded in lungs differs. Here the parasites enter endothelial lung cells. Lose flagellum. Round up tip to tail. Fuse into disc. Macro- and micro-nuclei move towards one another and fuse. Cell or cyst wall secreted : inside which single zygote nucleus divides into 2, 4, 8, 16 etc. Merozoites formed : cell ruptures into blood stream : parasites enter circulation : pass to periphery : develop into trypanosomes with most peculiar phenomenon : they are found either within, half in and half outside the R. B. Cs., or attached to the R.B.Cs., by their posterior poles. Recent workers however consider that Chagas' special lung cycle is inaccurate or refers to some other parasite present at the same time. Delanœ says they belong to Pneumocystis.

Anyhow peripheral blood becomes invaded with two types of typical adult trypanosomes which never apparently divide in peripheral blood, but whose supply is kept up from internal viscera : 1. free forms in plasma from schizogony cycle in muscle and neuroglia cells : 2. trypanosomes in or half in or attached to R. B. Cs., derived from schizogony cycle in lung.

Adult trypanosome forms seen in peripheral blood are about 20 µ long. In fresh films parasite shews a curious tumbling movement, unlike that of other trypanosomes. Cultures on N.N.N. successful, but best at 30°F. 12 months old culture proved virulent to mice.

Bug, T. megista, feeds on this peripheral blood. (Young larvæ used in Chagas' transmission experiments. N.B. ?? bearing on Conorhinus in kala azar ??.) Six hours after feed trypanosomes lost flagella, rounded up, became leishmania-like in type. After 23 hours parasites have passed into posterior part of midgut. Chagas believes that (1) those trypanosomes free in plasma pass into hind gut : multiply : become crithidial in type : bug's fæces infective : (2) trypanosomes in R. B. Cs. differ : pass through midgut wall, enter bug's body cavity : get to salivary glands : divide down into tiny crithidial forms which are injected by bug when feeding and so may infect new host. (Not confirmed by later workers). Chagas states that only in 1% of fed bugs does salivary infection take place. Believes usual mode of infection is crushing the fed bug into skin abrasion. Many Conorhinus shew natural flagellate ? crithidia, ? developmental stage of trypanosome in nature : and bed bug fed on such parasites retains infection. Torres claims to have infected kittens with disease by feeding infected bugs on them (excluding fæcal infection and crushing) : other workers, chiefly Brumpt, have failed. True life cycles still at issue: but Triatoma admitted as true transmitting agent.

Genus Trypanoplasma. Have two flagella: principal one passes posteriorly, attached by undulating membrane. In addition finer, free, anterior flagellum. Parasites are broad, leaf shaped, flat. Micronucleus band shaped, and very big, macronucleus usually situated close to it near anterior pole. Are parasites chiefly of marine fish (where transmission agent not known) and fresh water fish, transmission by leeches. In leech they multiply in the crop by binary longitudinal fission; injected by leech when feeds on second host. Kosselitz believes he has seen conjugation in leech, but doubtful. Whole group so far very little studied. Some trypanoplasmata described from invertebrates, e.g. the snail: and intestinal forms also discovered.

Literature.

The literature on Trypanosomiasis is enormous and impossible to summarise, but the student can gain a clear account from Manson's "Tropical Diseases." Also the account in Vol. II. of Byam's "Practice of Medicine in the Tropics" by Blacklock and Warrington Yorke is a good one, although other authorities do not agree with their claim that T. brucei and T. rhodesiense are one and the same parasite.

LECTURE 9.

THE SPOROZOA. THE GREGARINES AND COCCIDIA. THE COCCIDIA OF MAN. HÆMOSPORIDIA.

Phylum Sporozoa.

Are all parasitic; in fact this phylum has simply been created by lumping together all those protozoa which are parasitic and which reproduce by spore formation. Are blood or tissue inhabiting forms. Spore = resistant, seed-like body produced to withstand adverse conditions of environment outside host, and to carry infection from host to host via outside environment. Cf. plant and seed. As Sporozoa are all parasitic and get plentiful supply of nourishment from host by osmosis they have lost all organs for movement and for food capture. Use of term spore in reality a bad one, as rather meaningless: should substitute far more accurate merozoite for spore of the asexual, schizogony cycle: and term sporozoite for spore of the sexual, sporogony cycle. Books very remiss in nomenclature. Sporozoa divided into two big main groups:—1. Telosporidia: (telos = end): which grow into adult forms before starting to form spores: spore formation is end phase of life cycle. 2. Neosporidia: which commence to form spores whilst still actively growing and during active vegetative phase.

With the Sporozoa we come to sexual conjugation = syngamy, as a well established life process for the rejuvenescence of the parasite. Some Sarcodina, but not the entamœbæ, shew sexual conjugation: so do many flagellates, but not those parasitic in man. Here most essential at outset to clearly grasp nomenclature: may seem difficult, but is easily acquired and *must* be understood if life cycles are to be clearly followed. So:—1. The growing, living, undivided parasite in its vegetative or nutritional phase is called the trophozoite (trophos = food). Trophozoites multiply by asexual multiplication, by either binary or multiple fission, i.e. by schizogony (schizos = splitting): and from the moment the chromatin has started to divide the asexual schizogony cycle has set in, and dividing parasite now=schizont. The " spores " of the asexual, schizogony cycle=merozoites.

On the other hand, and especially as host is becoming resistant and parasite prepares to pass from this host into fresh one, sexual differentiation sets in. Certain trophozoites continue to grow without their chromatin splitting and become male or micro-gametocytes and female or macro-gametocytes. Male gametocyte produces one (or more usually many) male gametes : female gametocyte becomes transformed into female gamete. Thus from micro- and macro-gametocytes we get formed the actual cells of sexual conjugation, the micro- and macro-gametes.

Next microgamete fuses with and fertilises macrogamete. Test for true syngamy is that male and female pronuclei must fuse : i.e. chromatin from the two different sexes must unite : what happens to cytoplasm is irrelevant. Result of conjugation is a single cell, called at first the zygote (zygos=yoke) or ookinete (i.e. motile egg). This then comes to rest and becomes a motionless rounded oocyst (egg cyst). Oocyst next divides up into sporoblasts : which again divide up into sporozoites. Final product of the sexual cycle are the sporozoites which constitute infective agent for new host. Are termed sporozoites to distinguish them from the merozoites of the asexual cycle. Hence use of term spore to be deprecated. (E.g. you will find text books refer to malarial schizont as malarial sporont : term has no meaning, if it had it should be applied to the malarial oocyst). Coccidial and malarial life cycles should be studied in order to grasp the nomenclature.

Asexual cycle=schizogony-consists of trophozoite, schizont, merozoite, trophozoite. Sexual,=sporogony cycle-consists of trophozoite, macro- and micro-gametocytes, macro- and micro-gametes, syngamy, resulting in zygote, ookinete, oocyst, which forms sporoblasts, sporozoites, which become trophozoites again.

Telosporidia

Comprise three main classes :—

1. The Gregarinida, or " Gregarines " : trophozoite lives inside tissue cell, but adult parasite outside host cell.

2. Coccidia, parasitic in gut cells of both vertebrates and invertebrates : both schizogony and sporogony cycles.

3. Hæmosporidia, or blood-inhabiting Sporozoa, (cf. Hæmoflagellates). They shew asexual, schizogony cycle in vertebrate

host with sexual, sporogony cycle in invertebrate transmitting host, e.g., malaria in man and mosquito.

1. Gregarinida.

Parasites of invertebrates, worms, molluscs, insects etc, Young trophozoite lives in cell of intestinal mucosa. Grows in cell which becomes much distorted and distended and swollen and deformed. Next the parasite comes to lie partly in and partly outside the host cell. Attached to cell by tail until all nourishment sucked out of cell. Then drops out of cell and comes to lie free in gut lumen. Or may get into body cavity or even blood stream.

At this stage parasite has curious resemblance to minature tapeworm almost. Attached to exhausted host cell by epimerite, which has suckers ; behind this body of gregarine, "cephalont" as it is termed, divided transversely by partition into protomerite and deuteromerite. Also in body three layers of cytoplasm, outer epicyte forming cuticle and epimerite : thin intermediate myocyte containing myonemes ; and inner sarcocyte.

When gregarine drops into gut lumen it has a curious gliding motion, with shape alternately bulging and elongating, from contraction of the myonemes. Glides forwards in direction of its long axis : partly due to contraction of myonemes, partly to secretion, excretion and accumulation behind it of jelly like secretion : in fact in many ways recalls tapeworm.

As a rule Gregarines multiply only by sporogony : but one group, the Schizog egarines, shew schizogony cycle as well. Here simple multiplication by multiple fission. Most Gregarines however multiply only by sporogony.

Free trophozoite in gut lumen grows into gametocyte. At this phase special tendency for them to accumulate in clusters, whence name of the class, Gregarinida. Adult trophozoites become mature micro- and macro-gametocytes. Full grown male and female gametocytes pair and come together. Thin gelatinous cyst wall secreted around the pair. Nucleus of each gametocyte divides into daughter nuclei, which pass to periphery of the gametocyte. Each becomes a gamete, macro or micro according to sex of parent cell. Within each parent cell waste residual mass of waste chromatin and cytoplasm left. Next any or all septa etc. break down : gametes liberated inside cyst. Macro- and micro-gametes of most Gregarines are isogamous, i.e. similar in form and characters etc. Within cyst they join in pairs and sexual conjugation takes place. Result=zygotes=in number half the number of the original gametes. Each zygote becomes oval or spindle shaped, and surrounds itself with cyst wall=sporoblast. Each sporoblast now starts nuclear division : to form within itself 8 sporozoites : the latter being spindle shaped and packed like herrings in a barrel. At this stage we have :— oocyst or pansporoblast enclosing the sporoblasts : and inside each sporoblast are 8 sporozoites closely packed together. Either one of two things may now happen :—1. whole thing may rupture inside gut, setting free broods of sporozoites, which pass to gut wall, infect epithelial cells and become trophozoites. Or :—2. whole pansporoblast may be passed in fæces : get into water or soil, be swallowed by new host : in whose gut the whole thing ruptures and thus new host infected. Infection passes by contamination.

In case of Schizogregarines schizogony the main process by which gut wall becomes riddled with infection : one schizont producing up to 200 merozoites. With Eugregarines sporogony is mode of both continuing infection in same host and infecting others. More than 65 different genera of Gregarines, very big group. Place to see them best is seminal vesicles of earthworms (usually infected) or gut of flies or crabs etc.

2. Coccidia

Unlike Gregarines are very homogeneous group. Important to us because 1. They form a good introduction to malaria life cycles ; 2. five human coccidia known. Full life history of very few coccidia known. Are parasites of gut, liver or even body tissues of insects, molluscs, annelids and flat worms, birds, and all classes of warm blooded vertebrates including man. Rabbit coccidium, C. cuniculi, very fatal and often epidemic. Cat coccidium also very fatal, and at present much interfering with experimental dysentery work in Calcutta. Most important parasite to bird owners and rabbit breeders. Gut of almost every sparrow in India shews coccidiosis.

Best example is C. schubergi of centipede where whole life history known. Begins as minute ovoid trophozoite inside epithelial cell of gut (usually small, but often large intestine), nucleus single. Now grows, and as it does so infected host cell greatly swollen, distorted, deformed, cell nucleus pushed aside. Parasite nucleus divides into 2, 4, 8, 16 daughter nuclei=schizogony : these become merozoites : host cell becomes packed with merozoites : ruptures : merozoites set free, re-enter other epithelial cells : by degrees whole gut wall becomes riddled with coccidiosis and ulcerated and infected.

After 5 to 7 days when gut wall is riddled, parasite has exhausted nutrition from gut, conditions now adverse, commences sexual, sporogony, resistant cycle. Certain of the trophozoites now develop into gametocytes. Female or macrogametocyte within host cell becomes rounded, big, motionless body, full of food granules. Its nucleus throws off a little waste chromatin = polar chromatin : comes to rest in centre of cell : and macro-gametocyte becomes converted directly into one, big, single macro-, or female gamete. Drops out of infected cell into gut lumen.

Other trophozoites within cells become micro-gametocytes. Cytoplasm finely granular and no food reserve. Nucleus divides and multiplies into very numerous daughter nuclei. These pass to the periphery of the micro-gametocyte. Each becomes next transformed into a motile microgamete. Gametocyte leaves broken down host cell : and in the lumen of the gut breaks up into microgametes, each with two flagella. Thus we have now in the gut the big, round, motionless macrogametes, waiting to be fertilised and swarms of microgametes, rushing through fluid environment in search of them.

Male next penetrates and fertilises female. As it does so male leaves its cytoplasm behind : only chromatin entering the female. Nuclear spindle forms within female, on which chromatin of both male and female pronuclei become distributed and fuse. Result = zygote.

Zygote in gut lumen now secretes around itself very dense, thick, impervious cyst wall. (Coccidial oocysts when first discovered were not unnaturally mistaken for worm ova). At this, undifferentiated stage, now passed as oocyst in fæces : where, in the PASSED FÆCES FURTHER DEVELOPMENT GOES ON : (contrast entamœbic cysts etc.). Way to study coccidial oocysts in fact is to keep infected fæces as moist chamber preparations and study them from day to day. The oocysts are intensely resistant, resist exposure to almost everything : whence difficulty in ridding e.g. rabbit run of coccidiosis.

Nucleus of oocyst (in C. schubergi) now divides into 2, then 4 daughter nuclei. Cytoplasm aggregates around each and each becomes a sporoblast. In doing so there is usually left a small residual granular mass of unused up cytoplasm, usually loaded with fat globules and very refractile and characteristic of coccidial oocysts. Sporoblasts each have a thin delicate cell membrane of their own. Inside each sporoblast (for C.

schubergi) are formed by nuclear division two sporozoites, plus again, residual cytoplasm. Sporozoites are vermiform in shape. This is the ripe stage. Full oocyst=pansporoblast: containing 4 sporoblasts, each with 2 (total=8) sporozoites in it. Coccidia classified according to number and character of (*a*) sporoblasts and (*b*) sporozoites in each. Oocyst now swallowed by new centipede. In its gut whole thing goes to pieces. Sporozoites set free : invade epithelial cells of gut : new host infected. Infection via contaminative route. Life cycle of Coccidia first correctly worked out by Schaudinn and Siedlocki in 1897.

In passing compare this with Gregarine life cycles. Coccidia spend most of life cycle inside epithelial cells, Gregarines don't. In Coccidia male and female gametes are very different : in Gregarines they cannot be differentiated from one another. In Gregarines several zygotes are produced within the cyst wall : in Coccidia only one.

Five parasitic Coccidia of man known : as follows :—

1. *Isospora hominis.* As with all the human Coccidia here only the oocyst phases in the passed stool are known, and nothing of the life cycle : (which however, must approximate to above). Genus Isospora has 2 sporoblasts, each with 4 sporozoites. Discovered in fæces by Kjellberg, 1860. Presumably a parasite of the epithelium of the small intestine. First accurate account, Wenyon, 1915. (Previously described under 44 different names). Oocysts passed in fæces. Are usually passed before segmentation has begun. Elongate ovoid form, with narrow end drawn out into a sort of neck 25 to 33 μ long, by 12 to 16 μ broad. Wall thin, smooth, colourless, very resistant to fixatives etc. Outer hard procelain-like wall layer, inner thin delicate membranous layer. Contents aggregated into round ball studded with refractile ? fatty globules. Development in passed fæces in 2 to 3 days. 2 oval sporoblasts, each containing 4 sausage-like sporozoites result. Infection of second human host occurs when such ripe oocysts are swallowed. Aberrant forms noted by Wenyon, (*a*) degenerated oocysts which are much vacuolated and do not go on to develop sporoblasts, and (*b*) aberrant forms with only 1 sporoblast=8 sporozoites.

Isospora infection almost world wide, reported from almost all war areas. 70 cases of infection recorded by Wenyon, Egypt, Greece, Mesopotamia, Salonika and Gallipoli. Curious phenomenon with human coccidiosis that it seems to be harmless, (whereas animal coccidiosis is often fatal). Infection with I. hominis usually transient, 3 to 4 weeks only.

Connal, however, 1922, (Trans. Roy. Soc. Trop. Med. and Hygiene. Vol. 16. p. 223) records an interesting case. A laboratory worker was shaking a flask containing emulsion of fæces full of Isospora hominis cysts. Flask burst and contents got into his nose and mouth. Diarrhœa ensued six days later and lasted for 30 days. The diarrhœa was of a troublesome type, but was controlled by bismuth and characoal. Oocysts of I. hominis were found in the laboratory worker's stools for the last 8 days of the diarrhœa.

2. *Eimeria wenyoni.* Wenyon, 1915. So far only reported from four cases, but believe I have once seen it in Calcutta. Genus Eimeria has 4 sporoblasts, each with 2 sporozoites. Only fully developed oocysts as yet known. Mature oocyst spherical, diameter 20 μ. Outer thick, rugose cuticle (like Ascaris ovum). Inner thin cuticle, membranous. Sporoblasts oval, 10 by 7 μ size. Each has within it two vermiform sporozoites, lying head to tail, with residual mass between them, brightly refractile. Infection in all four recorded cases was very transient and very soon disappeared. No symptoms,

3. *Eimeria oxyspora.* Dobell, 1919. Only seen once in patient from Ceylon, examined by Dobell in London. Again fully developed oocysts only forms known. Large, spherical, 36 μ diameter. Outer rough coat, encrusted with fæcal particles, inner smooth lining membrane. 4 spindle shaped, long sporoblasts, each with 2 sausage shaped sporozoites. Also small bright oocystic granules=residue. Sporoblasts 31 μ long, 7½ μ broad, sharply pointed both ends. Walls of sporoblasts tough. Sporozoites blunt one end, pointed at other. Lie head to tail.

4. *Eimeria snijdersi,* Snijders and Dobell, 1921. Again only one case known, Sumatra, 1921 ?? identical with E. wenyoni. Oocysts very large, 45 μ. 4 sporoblasts, each with 2 sporozoites. Sporoblasts spindle shaped, 23 by 7½ μ. Each has inside it two comma shaped sporozoites, placed head to tail, and one or two bright refractile granules.

Site of human coccidiosis probably small intestine epithelium. Coccidia very fatal in animals etc., apparently relatively harmless in man. (??). In addition to above four, there has been described :—

5. ***Human hepatic Coccidium.*** Records here so scanty that description not possible. Discovered, Grubler, 1858 in liver of Paris quarryman with peritonitis. Other reports from

Giessen, London, Dressler, 1873. Silcock, 1890 reported case, drew outline drawings of oocyst, 20 μ diameter, no detail. Similar E. stiedæ of rabbit liver well known and fatal rabbit hepatic coccidium. Infection passes into liver via bile ducts, epithelium of which is heavily infected. Clinically infected liver looks as if riddled with T. B., local areas of liver necrosis plus inflammatory reaction. Oocysts formed in bile ducts and pass down into small intestine and so into fæces. Further information about this human parasite much needed, no evidence that it is the same as E. stiedæ, probably not the same but a specific parasite of man.

Coccidiosis often very fatal in warm blooded animals, e.g. E. zurni often causes fatal dysentery in cattle. But in birds often harmless, and in man oocyst formation seems to represent end phase of infection which usually seems to be relatively harmless (??).

3. Class Hæmosporidia.

Immense group of immense importance. Are simply those Sporozoa which inhabit blood : cf. Binucleata of flagellates. Special features :—1. are parasites living inside blood corpuscles, either R. B. Cs., or leucocytes, 2. have alternation of generations, schizogony and sporogony. 3. But, unlike Coccidia, with alternation of generations also goes alternation of hosts : e.g. malarial parasites : schizogony cycle in man, sporogony cycle in mosquito. 4. Oocyst forms are here NOT resistant, as they are sheltered within body of invertebrate host. Allied to Coccidia but here inhabit blood stream, not gut. Thanks to another celebrated and classical muddle by Schaudinn (see later) whole literature very confused, and only now becoming clear. Nomenclature hopeless : e.g. name " Plasmodium " applied to malarial parasites etiologically quite wrong. Plasmodium should mean either (*a*) a parasite which lives in blood plasma, which malarial parasites do not : or (*b*) should be confined to the true plasmodia of the Neosporidia, (see later). Name " Hæmamœba " of Minchin much more correct : but name Plasmodium has become so ingrained in literature that it is now impossible to dislodge it. Classification of group :—

1. Genus *Hæmogregarina.* Parasites of R. B. Cs., (in cold blooded animals), of leucocytes (in warm blooded vertebrates) : are not amœboid, do not produce pigment, male gametocyte does not " ex-flagellate " in fertilisation. Those of fishes, amphibia, snakes, reptiles etc. transmitted by leeches :

(?? those of snakes by ticks ??). Those of warm blooded animals by ectoparasitic arthropods, ticks, mites, fleas, etc.

2. Genus *Piroplasma.* Parasites of R. B. Cs. Form either amœboid or definite. No pigment produced. Male gametocyte does not ex-flagellate : parasites of generally very minute size. Known only in mammals and transmitting agent is always a tick. Here (vide later) are Orya fever and blackwater fever of man due to human piroplasmosis ?

3. Genus *Plasmodium* (or more correctly Hæmamœba). Are parasites of R. B. Cs., are amœboid. Produce characteristic melanin (or as much better termed hæmozoin) pigment. During fertilisation male gametocyte "ex-flagellates." Invertebrate host, so far as is known, and transmitting agent is always a mosquito : Culex for the Proteosoma sub-genus of birds : Anopheles for the plasmodia of man and apes etc. Include the malarial parasites of man.

4. Genus *Hæmoproteus* (or Halteridium). Grow inside the R. B. Cs., of birds : are amœboid : produce hæmozoin pigment. Male gametocyte ex-flagellates during fertilisation. Transmission by biting Hippoboscid flies. (?? Now that the life cycles of these parasites have been cleared up by work of Acton and Knowles for schizogony cycle and of Mrs. Adie for sporogony cycle ?? question as to whether they should not really be included in genus Plasmodiun ??).

5. Genus *Leucocytozoon* (vera). (as distinguished from the false, pseudo—" leucocytozoa " of warm blooded animals which are true Hæmogregarines). Parasites of certain greatly distorted and deformed host cells in blood, ? R. B. Cs., ? large mononuclear leucocytes, ? endothelial cells, ? true nature of host cell being doubtful. Met with so far only in birds. Are not amœboid, produce no pigment, but the male gametocyte ex-flagellates in fertilisation. Invertebrate transmitting host (probably) Hippoboscid biting flies.

(Only matter of importance in this lecture is to remember the Coccidia, since human coccidiosis certainly occurs in India. Also to note systematic position in protozoology of malarial parasites, and their close relations and cousins in the other Hæmosporidia).

Literature

The human Coccidia are very well dealt with in Chapter V. of Dobell and O'Connor's " Intestinal Protozoa of Man." For a splendid, but now somewhat out of date discussion of the genera, affinities and characters of the Hæmosporidia read Minchin's " Introduction to the Study of the Protozoa " chapter 15.

LECTURE 10.

THE HAEMOGREGARINES, PIROPLASMATA, PLASMODIA.

Hæmogregaringes one of the biggest groups of blood-inhabiting protozoa.

In blood of fishes, eels, snakes, amphibia, reptiles, toads, tortoises, crocodiles, lizards, mammals, cats, rats, mice, dogs, cattle. One even reported by Krempf, 1917, in spleen of a Chinaman : observation doubtful and not confirmed.

Haemogregarines of two types. (*a*) Those of cold blooded animals live in nucleated R.B.Cs. : (*b*) those of warm blooded animals live in mononuclear leucocytes. Haemogregarine apparently must live in neighbourhood of nucleus, some forms even, as H. bisecans, divide nucleus of host cell into two as they grow. First haemogregarine discovered that of frog by Chaussat in 1850, mistaken by him for a worm. Bentley in India, 1905, first discovered dog haemogregarine. Christophers dis covered H. gerbilli of Indian field rat, 1905, and worked out parts of life cycle. Patton gave good account of haemogregarine of palm squirrel.

Fullest known haemogregarine life cycle that of H. muris of the rat, may be taken as example. Young sausage-shaped trophozoite lives in liver cells inside a clear vacuole-like space. Parasite secretes a thick translucent capsule on inner surface of this vacuole, so that if it drops out of cell well defined vacuole still persists. Grows larger and distends infected cell, pushing host cell nucleus aside. Nucleus divides into two and halves pass towards poles of parasite : each half then divides again repeatedly, and finally liver cell becomes a pseudo-cyst filled with small sausage shaped merozoites, with small mass also of waste debris and residual cytoplasm. Cell now ruptures, merozoites scattered, some re-enter fresh liver cells : schizogony persists in liver.

Others however enter large mononuclear leucocytes and pass to peripheral blood : these become gametocytes. Sometimes not one, but 2, 3 or even more may be found in one large mononuclear leucocyte. Lie in cytoplasm close to leucocyte cell nucleus. Again lie in vacuole on inner surface of which is secreted tough, galatinous, translucent capsule: so that again, if parasite drops out of cell, well defined vacuole is left. Big oval nucleus consisting of aggregation of lumps of chromatin. By degrees rat's peripheral blood swarms with gametocytes.

Invertebrate transmitting host of H. muris is the rat mite, Lelaps echidenus. When mite sucks in infected rat blood, leucocytes dissolve in its gastric juice, gametocytes set free, and conjugate to produce a zygote. Zygote travels through gut wall into body cavity of mite, thence to muscles of thorax and to membrane investing salivary glands. Comes to rest, rounds up=oocyst. Oocyst grows rapidly in size, nucleus divides repeatedly to produce from 50 to 100 daughter nuclei, which pass to periphery of oocyst. Each then buds away with little attached cytoplasm as a sporoblast. The sporoblasts are oval.

Inside each sporoblast nucleus divides and clusters of daughter nuclei pass to each pole of sporoblast. Finally each daughter nucleus plus cytoplasm and investing cell membrane becomes tiny sausage shaped sporozoite. Final result is enormous oocyst=pansporoblast: inside it 50 to 100 oval sporoblasts: inside each of which lie about 16 tiny sporozoites packed like herrings in a barrel.

Rat searching through its fur eats such infected mites which get on to it from infected rats. In rat's duodenum mite is dissolved, pansporoblasts and sporoblasts set free, sporozoites liberated, enter villi of intestine, pass via portal circulation to liver: new rat thus infected. Infection also possible via bite of infected mite. Mites feed only at night, during day time leave rat and inhabit rat's run.

Many variations in other haemogregarine life cycles, but definite capsule always very characteristic of these parasites. In case of haemogregarines of cold blooded animals trophozoite lies as oval, fusiform or club shaped parasite inside capsule, with big, deeply staining nucleus. Inhabit either liver cells or nucleated R.B.Cs. In some species schizogony takes place in peripheral blood. Parasite grows, distends R.B.C., which becomes very pale and ghost-like. Parasite splits up into 8, 12 or 16 merozoites. Enlarged and infected R.B.Cs. caught in meshes of liver or bone marrow, and rupture, setting free merozoites which again enter fresh R.B.Cs. or liver cells.

As schizogony goes on differentiation sets in. Some schizonts break up into a few big so-called macromerozoites: others into a very large number of very small micromerozoites. These enter R.B.Cs. pass to peripheral blood, become gametocytes. Gametocytes are very characteristic, are like an incurved finger : if blood be examined in fresh state they leave the R. B. Cs. leaving behind dense capsule outlining vacuole. As they emerge they become club shaped, with bluntly pointed tail and round, blunt head : at head end is tiny beak-like rostrum projecting, from which passes down groove-like cytostome to end near nucleus. Nucleus big, round and very prominent. Conjugation observed in freshly shed blood but further life history not known. Mode of transmission not known.

H. canis of dog often incorrectly termed " Leucocytozoon canis." Term leucocytozoon should be strictly confined to the true leucocytozoa which are parasites of birds, vide later. H. canis inhabits certain immature mononuclear leucocytes which come from the bone marrow and have single, erratic nucleus. Site of infection is the bone marrow. Parasite lies in cytoplasm of these leucocytes, oval, not pigmented, inside very definite double capsule envelope, difficult to stain for this reason. Again if it leaves cell it leaves behind it well defined vacuole with outer shell of capsule. Multiplies in bone marrow by multiple fission, schizogony, till leucocyte becomes enormous pseudo-cyst, about 48 μ diameter, inside which lie about 30 merozoites. This now ruptures setting free merozoites which re-enter new leucocytes. Some become gametocytes and pass to the peripheral blood.

Sexual cycle of H. canis occurs in tick Rhipicephalus sanguineus. Female adult tick only feeds once during adult life, takes two to four days to feed, and when engorged drops off dog. In tick's stomach gametocytes leave leucocytes, and are elongated and worm like in shape and movement, often referred to as " vermicules." Both sexes appear identical, isogamy.

Enter into epithelial cells of gut wall and whilst lying there commence to divide up. Inside cytoplasm of epithelial cell is thus produced little pseudo-cyst containing from 4 to 8 little vermicule shaped gametes, derived from one gametocyte.

Two gametes next fuse, so one must be male and other female. Conjugation occurs as they lie side by side, and is isogamous. Zygote thus produced proceeds to throw out two waste bits of polar chromatin, swells in size, rounds up, becomes an oocyst with cyst membrane. When about 14 μ in diameter divides into sporoblasts. Final stage is big oocyst= pansporoblast, inside it sporoblasts, inside these the little sausage shaped sporozoites, about 12 to 14 in number.

Ripe pansporoblast now drops out of wall of gut into lumen of tick's gut, and presumably new dog becomes infected by eating infected tick, but exact mode of infection not known. Quite possibly the infection is handed on to the next tick generation, as with piroplasmosis, see later.

H. stepanowi of tortoise well known parasite. Three phases of its life cycle. Young sausage shaped trophozoite enters nucleated R. B. C. in bone marrow. Grows and by schizogony divides up into very numerous very small merozoites : cell ruptures, merozoites enter fresh R. B. Cs. Grow into schizonts again but this time schizogony only produces a few big merozoites : cell ruptures : merozoites enter fresh cells : this time grow and by schizogony produce many small merozoites. Thus two types of schizogony with alternate production of many micro-merozoites and few macro-merozoites. By degrees trophozoites pass in R. B. Cs. into peripheral blood and therein become gametocytes. Transmitting host is a leech which feeds on tortoise. Leech ingests blood : gametocytes liberated from R. B. Cs. in its intestine. Microgametocyte applies itself to macrogametocyte. Its nucleus divides into four : one of the four nuclei enters the female, the other three degenerating. Zygote and oocyst result, with remains of microgametocyte still attached to one pole of zygote. Oocyst invested with thin membrane. Inside zygote nucleus divides into 8 daughter nuclei, and 8 sporozoitis are formed. The oocyst now ruptures and sporozoites pass to and collect in the dorsal blood vessel of the leech. How they get back from this into the next infected tortoise is not known, possibly by ingestion ?

Haemogregarines a group of very great protozoological interest. Classical memoir by Sambon on haemogregarines of snakes well worth reading. Not however of human importance. In many ways they shew resemblances to the intestinal Gregarines.

Piroplasmata are small blood protozoa living in R. B. Cs. of warm blooded mammals and a very important cause of disease and death among them. Of immense importance in veterinary medicine. Pet dogs in India one and all suffer sooner or later from piroplasmosis : disease as easily diagnosed by blood examination and as easily treated as malaria in man : yet veterinary surgeons wont use the microscope, and thousands of valuable dogs suffer untold miseries for this reason and are improperly treated. Story of a vet. who did determine to use a microscope, but was found using the 2-3rds inch objective with immersion oil ! Microscope as important in veterinary as in human medicine.

First piroplasma identified was that of the sheep by Babes, 1880, and true systematic name of genus should be Babesia and not Piroplasma. Chief piroplasmata are :—

1. Of cattle. P. bovis in America, Australia, Asia, Africa : Anaplasma marginale : P. or Theileria parva, Rhodesia : P. bigeminum, Texas : P. divergens of Europe. Cause red water or haemoglobinuric fever of cattle.

2. In dogs P. or Babesia canis, world wide cause of tick fever of dogs : P. gibsoni of jackals, Madras.

3. In sheep P. ovis, Europe, Africa, W. Indies : cause of haemoglobinuric fever of sheep.

4. In horses Nuttallia equi, infects horses, mules, donkeys, zebras, Africa, Europe, Venezuela : P. caballi of Europe. Cause very fatal red water fever.

5. In rodents P. muris of the rat and P. avicularis.

6. In monkeys P. pitheci, cause of fever with 33% death rate.

7. In man Bartonella bacilliformis, cause of Oroya fever of S. America, but lacks confirmation and very doubtful.

This list only includes most important species. Destruction of animal life throughout the world by piroplasmosis so enormous that these parasites constitute economic problem of highest world wide importance. Generally speaking piroplasmosis may be either acute or chronic. Acute type causes high fever, sweats, rigors, terrific destruction of R. B. Cs. with haemoglobinuria, even haematuria, bilinuria, jaundice, profound anaemia, finally paralysis and death. Chronic type tends to kill after prolonged period of wasting, anaemia, jaundice and misery. Death rates terrific : red water fever of sheep 50%, East Coast fever of Rhodesian cattle 90%, Taxas fever of oxen 70%, etc.

P. canis as typical example of life history, parasite of tick fever, a disease which is nearly always fatal unless properly diagnosed and treated. Parasite discovered in 1895 by Piana and Galli Valerio, but full life history worked out by contributions from several workers, Christophers, Nuttall, Graham Smith.

If blood of infected dog be examined in fresh state we see many R. B. Cs. pale, enlarged and swollen. Contain little tiny pear shaped bodies with bright refractile centres. Some shew sluggish amoeboid movements inside the R. B. C. Other non-motile forms (gametocytes) seen also free. On staining each parasite is seen to consist of :—1. centrally placed nucleus staining with Romanowsky stains intense magenta red. 2. blue, faintly staining cytoplasm, with vacuole in centre and more condensed at periphery. 3. usually present a little bud of chromatin attached to main nucleus by fine thin chromatin strand.

Asexual life cycle as follows :—Youngest trophozoite is tiny ring, with central vacuole and tiny deeply staining nucleus lying inside (possibly on surface of) R. B. C. Grows and as it does so infected R. B. C. becomes enlarged, pale and ghost like. Parasite absorbs its HB, and has sluggish amoeboid movements within R. B. C. As parasite grows there buds off from the nucleus a small chromatin bud which moves away from it, but is still connected to it by a fine, thin chromatin strand. This stage lasts for long time and often seen in blood films. Vacuole next comes to centre of parasite, nucleus to one side, so again a ring form, but larger than before. Chromatin bud next divides into two : symmetrical masses of cytoplasm protrude from near these chromatin buds and carry the buds with them, the buds however still united by fine chromatin strand to main nucleus : trefoil shape appearance of parasite. Main nucleus now divides into two, and we get two pear shaped parasites resulting, lying in same R. B. C. but with tail ends joined. Each has nucleus near its pointed tail end, vacuole, cytoplasm, whilst the chromatin buds are breaking down. Finally they separate : two pear shaped parasites, each with single nucleus and vacuole lying side by side in the R.B. C. Curious type of binary fission with a specialised chromatin bud leading the way to cell division, rather recalling the action of the micronucleus in binary fission of Herpetomonas.

Infected R. B. C. now breaks down, parasites liberated, enter fresh R. B. Cs. Modifications of this schizogony cycle may occur. Instead of binary fission we may get nuclear division into 2, 4, 8, 16 with formation of rosette of 16 merozoites in R. B. C., similar to that for Proteosoma, see later. Or growing piroplasma may leave the R. B. C., develop two basal granules from which two flagella arise, and a motile form living free in the blood results. These forms very rare but have been described by several most careful workers. The curious character of the chromatin budding in binary fission, and the existence of these flagellate forms, whose function is unknown have led many workers to consider that the Piroplasmata should be placed among the Haemoflagellates and not among the Haemosporidia at all.

Transmission of tick fever from dog to dog a marvellous example of adaptation to natural environment. Would have delighted Darwin had he lived to know of it. Transmitting tick varies in different countries, Haemophysalis leachi in Africa, Rhipicephalus sanguineus in India, Dermatocentor reticulatus in Europe. Adult female tick feeds only once during life time, takes two to four days, when engorged drops off dog on to ground. As a result 100% of ticks feeding on infected dogs get infected. In tick's mid gut the pear shaped parasites, which we must regard as gametocytes, leave the R. B. Cs., lie free and swell up into round forms, 4 to 5 μ diameter. Cytoplasm divides into two bits, one piece without chromatin, one piece with. Latter turns round to form sort of club shaped tail to gametocyte. These forms next elongate, become vermicule like in shape, pass through gut wall of tick's stomach, get into body cavity, pass to the tick's ova, enter them, and THUS INFECT THE OVA AND THE NEXT TICK GENERATION, being present in the ovum when it is fertilised by the spermatozoon.

Infected ova now laid by the tick on ground. Ovum becomes larva : larva feeds once on dog, but is not infective. Larva becomes nymph. Nymph feeds once on dog, but is not infective. Meantime in the embryonic tissues of the tick larva and nymph the vermicule like gametocytes become small, round up and are directly transformed into gametes. These conjugate, zygote produced, becomes oocyst, within which develop sporoblasts and inside them sporozoites. End phases of sporogony cycle just like those of malarial parasites in mosquito, so need not go into detail. The salivary glands of the second tick generation become crammed with piroplasma sporozoites, which travel to them after rupture of the pansporoblasts. Sporozoites are tiny, oval, pear shaped forms, and when the tick is ADULT it feeds, once only, on a dog. As it does so the sporozoites pass down its salivary ducts, are injected into the dog, which is thus infected. As tick takes two to four days to feed 100% of infected ticks will infect the dogs on which they feed.

Note the extraordinary beauty of this adaptation of the life cycle to biological necessities. Parasite must pass from dog to dog through ADULT tick. But adult tick feeds only once. Hence if infection is to be passed from dog to dog via tick Nature had got to work out some mechanism of hereditary transmission : hence the curious slow, delayed phase of

parasite development through the ovum, larva and nymph stages and delayed conjugation, to be succeeded by rapid invasion of the salivary glands when the salivary apparatus becomes mature.

All piroplasmata transmitted by ticks. In some the oval forms when ingested by the tick may shew pseudopodial activity and throw out ray like pseudopodia or assume stellate forms in tick mid gut. Zygote may be vermicule like or globular in shape.

Treatment of canine piroplasmosis we owe to Nuttall and Hadwen. Inject dog intravenously in saphena vein with 2% solution of trypan blue in saline, giving 0.02 gm. per kilo weight of dog. Dog literally turns blue, parasites killed, drug subsequently gradually eliminated. Dose may have to be repeated two or three times, but usually once sufficient. Every pariah in India gets piroplasmosis sooner or later, usually sooner, and pet dogs infected from their ticks.

P. gibsoni of jackal very similar, but rings in R. B. Cs. very minute. Cattle piroplasmata very important group. P. bovis of sheep very minute, almost bacillary in size and shape. Tick Ixodes reduvius spreads it. Genus Anaplasma ultraminute, look merely like little tiny chromatin dots on margins of infected R. B. Cs. Many workers refuse to admit this genus as true one, and many so-called Anaplasmata are artefacts : but A. marginale probably a true parasite. Infection spread by tick Rhipicephalus decoloratus. Causes haemoglobinuric fever of cattle or sheep.

Theileria parva ultra-minute, almost bacillary in size, inhabits both R. B. Cs. and lymphoid tissue. Causes acute oedemic fever of cattle in Rhodesia. Infected R. B. Cs. shew parasites arranged in cruciform cluster of four. Multiplies in R. B. Cs., lymphoid tissue and cells of spleen and lymph glands. In some parts of Rhodesia all the cattle have died of disease, grass is full of infected ticks of next generation, and if healthy cattle be driven across these tick belts they get piroplasmosis and die. Grave S. African problem of how to rid grass lands of ticks. P. bigeminum causes Texas fever of oxen. Pear shaped parasite with characteristic binary fission forms, whence name bigeminum. This parasite has been successfully cultivated on artificial media.

P. bovis of sheep the cause of red water fever : in acute form of disease 90% of the R. B. Cs. infected, mortality 50%, hereditary transmission via Ixodes and Margaropus ticks. Nuttallia equi causes acute biliary fever of horses : small and large pear shaped forms seen in blood, large and small spherical ones, rod like forms and very rarely free, motile, flagellate forms. Transmitted by blue horse tick, Rhipicephalus decoloratus. In monkeys P. pitheci infects Cercopithecus monkeys, pear shaped, 2 to 3 μ long, 1½ to 2½ μ broad : of 12 monkeys found infected by P. H. Ross 4 died with high fever.

Bartonella bacilliformis supposed to be the parasite of Oroya fever of man, in Peru and S. America. Discovered by Barton, 1901, and reported by Harvard School of Tropical Medicine Commission, 1913, as true cause, but doubtful. Hitherto two diseases confused together : Verruga peruviana or Peruvian wart which is a granulomatous skin lesion : and true Oroya fever, Carron's fever—human piroplasmosis. Acute fever with profound anaemia. Carron proved that virus was persent in peripheral blood : inoculated himself with blood from patient, died 39 days later from most acute type of the disease. Parasite said to be of two types : in the R. B. Cs. tiny motile bacilli-like rod-shaped piroplasmata, 1½ to 2½ μ long, with one chromatin particle at each end of rod. Other form found in endothelial cells of spleen and lymph glands and is tiny round from only about one μ in diameter. Strong and Sellards describe these forms as multiplying inside infected cells by multiple fission and producing immense broods of merozoites which may re-enter fresh endothelial cells or enter R. B. Cs. and pass to peripheral blood. So far all experimental work with ticks and at attempted cultures on artificial media have had negative results. Very doubtful to what extent these findings may not be artefacts, and not true parasites at all. Parasite found by several workers, but needs confirmation. No reason at all why human piroplasmosis should not exist, however.

Here note that, especially in tropics artefacts are especially numerous in stained films and may be mistaken for parasites, especially for piroplasmata. Dont diagnose piroplasma without seeing 1. definite outline. 2. red chromatin. 3. blue cytoplasm. An excellent account of the " Fallacies and Puzzles of Blood Examination " by Balfour in Vol. A of the 4th Report of the Khartoum Wellcome Research Laboratories deals with the subject splendidly and should be read by all laboratory workers in tropics. Corpuscles in blood not all in same state of health or age, and may stain badly : or deposit of stain may be mistaken for piroplasma : yeasts not uncommon in blood films in tropics : X bodies in degenerated glass slides, especially in slides formerly in alcohol where spirit has evaporated. Also met with in cover slips and have known them mistaken for protozoal cysts. Castellani's Toxoplasma pyrogenes, (p. 489 of Castellani and Chalmers) very like artefact: many so-called Anaplasma are almost certainly artefacts: Ovoplasma of de Raadt, 1913, actually stated to have no chromatin, almost certainly an artefact etc.

Plasmodia.

Members of the genus Plasmodium live in R. B. Cs., and multiply by schizogony inside them: produce haemozoin pigment: during fertilisation the male gametocyte ex-flagellates: and they occur as parasites of man, warm blooded mammals and birds. The group includes :—

1. The malarial parasites of man. 2. The Plasmodia of monkeys. 3. The subgenus Proteosoma of birds. and 4. a few other Plasmodia such as P. canis of the dog, P. vassali of squirrels etc.

In dealing with malaria will try as far as possible to avoid any aspect of the subject other than the protozoological: but here emphasise the ABSOLUTE NECESSITY FOR ANY MEDICAL MAN IN JUSTICE TO HIMSELF AND TO HIS PATIENTS TO INCESSANTLY USE THE MICROSCOPE in medical practice in the tropics. The man who doesnt make a friend of his microscope is lost. He begins by treating diseases, then he loses the power to diagnose them, and commences to treat only symptoms. However big a fortune he makes he ends by being a quack pure and simple. A medical man in India who attempts to practise without using a microscope is like the captain of a ship who puts to sea without a compass and sextant.

There are three great malarial parasites of man. There may be others, but sufficient evidence not yet brought forward to confirm these others as indepedent species. They are :—

1. Plasmodium vivax: the parasite of B. T. malarial fever. As shewn by Acton, 1918, this is THE parasite of relapsing malaria, and much more resistant to quinine than is P. falciparum.

2. Plasmodium malariæ, the parasite of quartan malarial fever. This parasite is also especially associated with relapses, and is the most difficult of all malarial infections to eradicate. Distribution of quartan fever in India: draw a line from Delhi to Madras. Quartan malaria occurs East but not West of it.

3. Laverania malariae or Plasmodium falciparum, the parasite of malignant tertian malaria and above all the parasite of epidemic malaria. Its gametocytes are crescentic, and many workers consider that it shews such marked differences

from the others that it should go into a new genus Laverania, (see later.) This is the most readily amenable of all three to quinine treatment.

Practical Notes. The Romanowsky Stains.

The student will never learn how to recognise and identify malarial parasites unless he can make good blood films and stain them properly.

1. Preparation of a blood film. Take a perfectly clean glass slide. Scratched, dirty, and frosted slides are useless. If at all greasy flame it and then polish with a soft cotton cloth, e.g. clean handkerchief. Take a second similar slide selected as having perfectly smooth ends with no rough or jagged edges by passing the thumb along the end. Under water with a sharp pair of scissors cut off the corners : this makes a spreader. Its edge must be perfectly smooth. The best place to prick the patient is NOT the pulp of the finger which is painful, but the back, just near the root of the nail. The hypodermic needle used for pricking should be mounted in a cork which forms the stopper for a small phial filled with acetone 40 : alcohol 60 parts. The needle can be taken out of this and flamed before use.

Take the slide on which the film is to be spread and, having punctured the patient's finger, hold the slide upside down and just touch the exuding drop of blood, so as to get a small drop on the slide about half an inch from its end. Turn the slide the right way up on the bench. Touch the drop of blood with the edge of the spreader slide : wait till the blood flows between spreader and slide evenly, and then draw the film, drawing the blood after or behind the spreader and not pushing it in front of it, a procedure which greatly distorts the cells. Hold the spreader at an angle of 45°. The smear should be smooth, even, with a good leucocyte edge. See Sir A. Wright, "Technique of the Teat and Capillary Glass Tube," p. 69, for a splendid account of how to make films and the errors to be avoided in doing so. If the pressure used in spreading be too heavy the film will ribbon and the cells be distorted : if spreader be held too vertically the film will be too thick : if the slide be greasy film will be bad. Many books recommend examining fresh unstained blood, but method even more open to errors than is examination of stained films. Let the film dry before staining.

For staining for all blood-inhabiting protozoa the stains most universally applied are the Romanowsky ones.

(*a*). *Leishman's stain.* Take a perfectly clean drop bottle. Give it a rinse with Merck's purest methyl alcohol. Drop into it two of B. W. and Co.'s Soloid Leishman stain soloids. Add 20 c.c. of Merck's purest methyl alcohol, acetone-free. Turn the stopper so that none will come out and shake UNTIL EVERY PARTICLE HAS GONE INTO SOLUTION. This last the most essential step of all. (Or if powder be used dissolve 0.15 gm Leishman's stain in 100 c.c. Merck's methyl alcohol by grinding it to complete solution in a glass—not porcelain—pestle and mortar. Put it into perfectly clean drop bottles). Next place the drop bottle in the warm incubator 24 to 36 hours for the stain to ripen. The two essential steps are to ensure complete solution of every particle of stain and to let it ripen before use. Personally recommend using two drop bottles in rotation : one in use : other with freshly prepared stain in incubator.

Lay slide flat on staining rack, prepared by fixing glass rods across a Petri dish with plasticine. Pour on Leishman's stain from drop bottle to cover film. Leave whilst " 15" is slowly counted, e.g. 20 seconds or so : the methyl alchool in the stain fixes the film. Add an equal number of drops of distilled water : mix THOROUGHLY. Stain for 10 to 15 minutes. Wash (*a*) in distilled water no. 1. (*b*) soak in distilled water no. 2. for about a minute to differentiate until the film is pink.

Leishman is the readiest of all stains for general blood films.

(*b*). *Giemsa's stain* The film must first be fixed. Cover it with Merck's purest methyl alcohol poured on from a drop bottle. Leave for one minute. Wash thoroughly in distilled water. The film is now ready for staining.

To prepare Giemsa's stain :—Dissolve with aid of glass pestle and mortar 0.3 gm. of Azur II eosin and 0.08 gm. of Azur II in 25 c.c. of Merck's or purest anhydrous glycerine. Add 25 c.c. of Merck's purest acetone-free methyl alcohol. Place overnight in the warm incubator.

For use take 10 c.c. of fresh distilled water. Add to it ten drops of Giemsa's stain from a drop bottle and mix. Cover the slide when fixed with this and stain for 20 to 30 minutes. Differentiate in distilled water, two changes, blot.

Giemsa's stain generally preferable to Leishman's.

(*c*). *Panoptic Stain.* (often gives very pretty results). Stain first by Leishman's method as above. Wash in distilled water. Pour on diluted Giemsa as above : stain 30 minutes or longer. Wash in distilled water. Differentiate in 1 in 1000 acetic acid, followed by thorough wash in tap water. Blot.

(*d*). *Original Romanowsky stain.* Troublesome but gives excellent results, and specially recommended by Christophers for use in large laboratories and where many slides have to be stained and for class work. Slides should be fixed by methyl alcohol as above. Two stock solutions needed :—

Solution (*a*). Medicinal methylene blue, 1 gm : sodium carbonate 0.5 gm. : Distilled water 100 c.c. When made place in the warm incubator to ripen, 2 to 3 days. Should be purple colour.

Solution (*b*). Eosin, extra B. A. water soluble. One gm. to distilled water 100 c.c.

To stain fixed slide : dilute both (a) and (b) each with 20 parts of distilled water and mix equal parts of both diluted stains. Pour on to slides and stain for 20 to 30 minutes. Soak in distilled water to decolourise.

There are many points about the Romanowsky stains of importance. Deeper staining may often be obtained if 1 in 1000 potass. carbonate be used in place of distilled water to dilute the stain with. The use of a rocker to keep slides moving whilst staining will avoid deposit and is very advantageous. If there is much deposit on the slide, especially after Leishman staining, give a rapid rinse with rectified spirit followed by thorough wash with distilled water. The film should be pink, not blue. If too blue soak for a few seconds in 1 in 1000 acetic acid, followed by thorough wash in tap water.

Romanowsky stains are compounds of two anilene dyes, eosin, extra B. A., water soluble, and medicinal (not pure) methylene blue. Latter contains oxidation compound called azur. When solutions of eosin and of medicinal blue mixed together we get whole range of loose chemical compounds, e.g. Azur I eosin : Azur II eosin : etc. These possess different affinities for different elements in nucleoplasm and cytoplasm respectively, and hence give differential staining. Cytoplasm of protozoal blood parasites stains, as a rule, pale clear, Cambridge blue : their chromatin deep ruby, magenta red. Eosin a cytoplasmic stain, methylene blue a chromatinic stain, other compounds intermediate.

The student should now take every opportunity to examine both normal and malarial blood films and to do differential leucocyte counts, (see later.) The frontispiece to Panton's "Clinical Methods" gives an excellent plate of the normal and abnormal cells met with in blood films as stained by Romanowsky methods and should be studied. It is most essential to know these. Briefly as under :—

1. The R. B. C. stains pink. As eosin is a dichroic stain may however appear greenish. Nucleated R. B. Cs., normoblasts and megaloblasts have a purple violet nucleus and pink cytoplasm, latter fact differentiates them from lymphocytes.

2. Polymorphonuclear leucocytes shew violet red nuclear network, very little or no colour in cytoplasm, and are studded with faintly pink fine granules. Nucleus multilobed.

3. Lymphocytes, both small and large, have deep violet nuclei and cytoplasm staining clear blue varying from pale to rather deepish blue. Nucleus spherical, cytoplasm scanty.

4. Basophile leucocyte or mast cell, nucleus violet reddish, and large granules, staining deeply blue black. Nucleus often or usually bilobed.

5. Coarsely granular eosinophile leucocytes, nucleus horse shoe shaped or bilobed, deep violet : cytoplasm packed with large granules staining deep brick red.

6. Blood platelets deep ruby red to violet colour with scanty rim of bluish and irregular contour. For some reason or other in occasional films they stain with a capsular, spherical, clear cut outline.

7. Large hyaline mononuclears have large globular nucleus, stains deep violet, much very pale blue cytoplasm. Transitionals are cells with more or less horse shoe shaped nucleus intermediate between 7 and the large lymphocyte, but for all practical purposes grouped with 7.

8. Turck's irritation cell is a transitional large mononuclear with the cytoplasm staining intense blue.

In nos. 3 & 7 we often meet with more less fine chromatin granules shot out from nucleus and lying in cytoplasm : have violet deep staining reaction, and must be carefully distinguished from malarial haemozoin.

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LECTURE 11.

MALARIAL PARASITES, CYCLES IN MAN.

PLASMODIUM VIVAX, B. T. parasite, so called from its vivacious cytoplasm. Discovered, Grassi and Feletti, 1890. Begins life as a small ring (i.e. young trophozoite) applied flat on the discoid surface of the R. B. C. Youngest ring forms are round or oval, with marked vacuole, flimsy cytoplasmic ring, and chromatin as rounded dot or globule, either in thinner side of ring, or just within the vacuole. 2½ to 3 μ diameter, or little larger, ¼ to ⅓ diameter of R. B. C.

Ring quickly grows into bigger trophozoite. Growing forms characterised by :—1. flimsy streaming cytoplasm as seen in fresh drop are very actively amœboid, sending pseudopodial streamers throughout infected R. B. C. In stained film this very active movement leads to :—2. fact that in stained films at this stage hardly any two parasites look alike : all sorts of shapes and forms, multiplicity of forms, tennis racquet, angular, oval, etc. At about 14 to 16 hours of age the parasite now gets into the R. B. C. and is henceforth intracellular. At the same time hæmozoin pigment is formed by the parasite=product of digested HB. of R. B. C. Pigment in B. T. parasite is yellow brown, angular particles or rodlets, seen in fresh drop parasite is actively amœboid with yellow brown pigment moving about, carried by pseudopodial currents. Haemozoin grains in B. T. parasite fairly evenly distributed throughout cytoplasm.

At same time parasite exerts and causes certain changes in the infected R. B. C. which becomes enlarged, and very pale, often a mere shadow. At an early stage Schüffner's dots appear. They are not infrequently absent in some cases, they are the first thing to fade in old Leishman stained slides, are sometimes seen whilst parasite is still at ring stage, sometimes not seen till much later. Exact nature doubtful : but probably are crinklings of surface membrane of R. B. C., ? points at which parasite pseudopodia have attached themselves and then been withdrawn. As the parasite matures Schüffner's dots, at first visible as a fine, pink staining ; stippling may become very prominent and marked.

Sooner or later parasite chromatin divides, at first into two. Time of this division may very : rarely young rings may shew divided chromatin : more usually the parasite has become large and nearly fills the R. B. C. before schizogony sets in : may be as old as 40 hours or so. The moment chromatin division sets in the form present is known as a schizont. (schizos=splitting, of chromatin).

In B. T. infections we may get 2 or even 3 rings in same R. B. C.=multiple infections, perhaps in some 1% of infected R. B. Cs. Multiple infection in R. B. Cs. much rarer in B. T. than in M. T.

Schizont still grows and infected R. B. C. becomes even larger and paler, 9 to 10 μ or more. Now becomes less amœboid and more rounded, and chromatin divides into 2, 4, 8, 12, or more daughter nuclei. Usually is believed that B. T. parasite produces about 18 to 20 merozoites, but recent careful count of 200 such schizonts at Calcutta shews only from 10 to 19, with an average of 14. After 40th hour cytoplasm begins to aggregate around each daughter nucleus, and the merozoites commence to become separated off, and each invested by delicate cell membrane of its own. Thus we may get stages at which we have fully developed merozoites plus part of the parasite still not fully divided.

At 48 hours the schizont has matured into the fully developed rosette. Ripe schizont rosette consists of a grape-like cluster of merozoites : and a small excentric mass of residual cytoplasm containing the accumulated pigment. 14 or more merozoites are usually present. The infected R. B. C. now ruptures : merozoites are set free. The patient has a rigor, due to liberation of toxins by parasite ? anaphylactic in origin, (see later). Free merozoites are little ovoid bodies with single nucleus. At time of ruptutre of rosette pigment is set free and is phagocytosed by macrophages and large mononuclear hyalines.

About the 8th to 9th day of fever, 8 days after first rigor, patient commences to become resistant, parasite prepares for sexual cycle, gametocytes are produced. Asexual cycle gradually dies out, leaving only gametocytes in peripheral blood.

Young B. T. gametocytes develop from ordinary ring forms. Early gametocyte is a rather solid ring with little amoeboid activity. Grows more slowly than do schizonts. Shape remains more globular. Female has very granular cytoplasm and rather heavy pigment, and male lighter cytoplasm and finer pigment. Sexual forms usually more pigmented than are asexual forms.

Full grown macrogametocyte very striking object, frequently seen in B. T. films. Females as a rule more numerous than males. 12 to 14 μ diameter. Lie in pale, enlarged, almost invisible R. B. Cs. which may or may not shew Schüffner's dots. Much deep blue cytoplasm, considerable granular hæmozoin pigment, large nucleus like a tightly wound up string of deeply staining chromatin, lying to one side of parasite in pale area which=remains of vacouble.

Microgametocyte, also a round body in enlarged and pale R. B. C. stains badly. Cytoplasm pale greenish grey colour. Pigment lighter and more yellowish than in females. Chromatin present as loosely wound and badly staining skein stretching more or less across equator of parasite like a cholera belt.

Gametocytes persist in peripheral blood for some time, certainly some days, possibly some weeks after asexual cycle has died out. Further development is in mosquito : see next lecture.

As a rule in B.T. cases seen early all parasites are at same phase, e.g. all young rings, or all rosettes. Later some parasites lag in development, especially if patient has taken insufficient doses of quinine. As infection gets established therefore we get films shewing everything simultaneously, young rings, growing trophozoites, schizonts, rosettes, gametocytes : and this is characteristic of B. T., and to a less extent of quartan infections.

PLASMODIUM MALARIÆ, Quartan parasite. Discovered by Laveran, 1881. In India especially in Assam, Dooars, Bengal. Schizogony cycle 72 hours. R. B. C. is unaltered, neither pale nor enlarged, and no dots : in fact is usually a little shrunken if anything. In fresh drop quartan parasites have special opaque, white, refractile look. Little or no amœboid activity, and pigment intense and very marked, is THE pigmented parasite. Parasite always compact, globular with dense outline.

Youngest rings lie obliquely on surface of R. B. C. Typical signet ring of text books. Pigment often seen even in early ring.

Growing trophozoite starts to form pigment at once, as dense black brown chunks, bars, rods. Quartan an immobile parasite with dense pigment : B. T. a very actively motile, flimsy parasite with scanty yellowish brown pigment. Early trophozoite tends to stream across cell like a comet, with chromatin mass in tail. Ribbon forms across cell common. Schizont reaches maximal size at 60th hour. Ring probably penetrates into R. B. C. about the 20th to 24th hour. Nearly ripe schizont is large, round, deeply pigmented body in R. B. C. of normal or small size, staining well. Chunks and bars of blackish haemozoin, and 5 to 8 chromatin masses. Pigment cluster at first excentric : later moves to exact centre of rosette.

Final rosette is typical "daisy" of text books. 6 to 12, usually 8 or 9 merozoites arranged symmetrically around central dense pigment agglomeration and waste cytoplasm residue. Rosette usually typical daisy : but may get it a little asymmetrical and with two pigment clusters in place of one. This now ruptures at 72nd hour, and free merozoites enter fresh R. B. Cs. Merozoites a little larger than B. T. ones.

Gametocytes begin as small globular rings. Quartan gametocytes are rare findings. Macrogametocyte very striking object. Spherical, compact globular form not quite filling unenlarged and normal R. B. C. Cytoplasm stains deep blue, tightly wound chromatin mass lying in pale area. Rather resemble those of B. T. forms.

Quartan microgametocyte very rare finding, but also is THE most deeply pigmented malarial form known. Pigment present (*a*) as a wide loose band across equator of parasite, (*b*) as a dense excentric cluster. Chromatin present as loosely wound, badly staining skein like loose cholera belt across parasite, lying over pigment band. Cytoplasm stains dirty grey green.

Further sexual cycle in mosquito. In quartan infections all phases of schizogony, rings, growing trophozoites and rosettes are often seen simultaneously : but gametocytes are rare : in fact better descriptions of them are wanted.

PLASMODIUM FALCIPARUM, or LAVERANIA MALARIÆ. The parasite of M. T. malaria. Many workers consider that this parasite should be placed in a separate genus, Laverania. Their reasons are (a) the M. T. gametocytes are crescentic and possess a very definite cell envelope or membrane of their own ; (b) schizogony, instead of taking place regularly every 48 hours, tends to be a far more continuous process than is usually recognised ; (c) schizogony takes place in the internal viscera and not in the peripheral blood stream ; (d) the parasite shews a special tendency to cause blocking of internal capillaries, as in the brain and intestines (whence its malignant character). These authorities accordingly propose to place the M. T. parasite in a new genus and call it Laverania malariæ. L. falciparum incidentally would be a better name.

But P. falciparum is the more familiar term. Parasite was discovered by Grassi and Feletti in 1890.

Youngest ring forms are practically dots of chromatin with trace of cytoplasm applied to the margin of R. B. C.=accolé form. 1¼ to 1½ μ. Others may be larger, even as big as B. T. rings. Rings are sharp and regular in outline. Young rings ⅙th to ⅐th diameter of R. B. C. Chromatin rather as tiny curved bar than as granule or blob. EARLY DIVISION OF CHROMATIN INTO TWO IN VERY EARLY RING STAGE is characteristic. Multiple infection of R. B. Cs. each shewing 2, 3, 4, 5, or even 8 rings apiece common and characteristic.

Infected R. B. C. does not change. Young rings in M. T. infections tend to all look the same and to be rather uniform, contrast B. T. At first infected R. B. C. not altered : later Maurer's (or more correctly Christophers') dots appear. Are irregular cleft-like markings in membrane of R. B. C., stain brick red and deeply : much coarser than Schüffner's dots. Are often however NOT seen in M. T. infections.

Last phase here seen in peripheral blood films (with very rare exceptions) are young rings with 2 chromatin dots. Next whole schizogony cycle is transferred to the internal viscera, and is not found in the peripheral blood. This leads to special malignant character of M. T. parasite. Capillaries of brain, gut, liver etc. get blocked with schizogony rosettes of M. T. parasites with as a result cerebral, intestinal and hepatic symptoms. In visceral and portal blood stream schizogony proceeds. Occasionally in fatal and fulminant cases may be seen in peripheral blood. Adult schizont small, 4 to 5 μ, occupies only half infected R. B. C. Number of merozoites varies much, usually said to be 8 to 14, but said that in culture they produce 32 (?? double infection, two schizonts in same R. B. C.: vide later). Usually held that all rosettes mature at same moment, so that rigor every 48 hours : but this not strictly true. Fact is that schizogony is more continuous and merozoites are being liberated more irregularly than with B. T. parasites. Not nearly as uniform a cycle. This corresponds with the quite irregular temperature charts so often seen in M. T.

M. T. gametocytes are the well known "crescents." Term is really a misnomer : more sausage shaped than crescentic, and poles not pointed. Typical crescents are from 9 to 14 μ long, 2 to 5 μ broad. Early gametocyte forms (only seen in viscera) are egg shaped in R. B. C., with stellate chromatin. Grow into adult crescents : which are seen to be more numerous in peripheral blood films than in e.g. spleen smears. Occupy whole of infected R. B. C., the remains of which are seen usullay on concave side of crescent, like stretched bow string.

Male crescent sausage shaped, shorter and fatter than female. Little and very pale blue cytoplasm. Pigment as fine granules, blue black, occupies middle two thirds of crescent. Nucleus=loosely wound skein, badly staining, occupying centre of crescent and shewing amid pigment spreading towards both poles.

Female crescent=macrogametocyte, more pointed at ends, thinner and finer than male. Cytoplasm stains more deeply blue. In centre is compact and aggregated clump of blue black pigment. Also in centre (in spread films very often a little displaced and peeps out from pigment mass) is tightly wound, deeply staining chromatin ball : in exact centre of crescent with pigment heaped over it. Usually number of females exceed number of males, but this not always true. Crescents can be easily counted by method similar to that for counting number of total leucocytes per c.mm. of blood : and ratio of sexes determined.

In addition to remains of R. B. C. stretched over crescent, crescents possess very definite cell envelope or capsule of their own, vide later. It becomes prominent only when the blood is shed.

Sexual cycle will be dealt with in next lecture.

Stephens in 1914 described what he regarded as new malarial parasite, P. tenue : resembles P. falciparum, but is very flimsy and amoeboid. Sinton, 1922, agrees with him and says that as compared with P. falciparum, P. tenue is much more amoeboid, its haemozoin lighter and more yellowish, later schizogony phases are seen in the peripheral blood, and it shews very much chromatin in in early forms. Most authorities however consider that P. tenue is only an aberrant type of P. falciparum. Stephens also 1922, describes a new parasite, P. ovale which resembles P. malariæ but lies in an unenlarged R. B. C. shewing Schuffner's dots. His plate however suggests a mixed B. T. and quartan infection and P. ovale lacks confirmation.

Other workers describe parasites with only 24 hour cycle : viz. P. praecox (precocious parasite) with schizogony every 24 hours ; P. immaculatum, the immaculate parasite, sub species of P. præcox, but has no pigment. Craig in South America describes malarial parasite with special tendency to cause œdema. None of these confirmed. Better,—at least until further evidence is forthcoming,—to accept only the three well recognised B. T., quartan and M. T. species. Quotidian fever and rigors in malaria easily explained by B. T. plus M. T. infections in same patient, broods maturing on alternate days, or B. T. plus B. T. : two lots maturing on alternate days : or, B. T. plus quartan : etc. 1923 Calcutta case with films shewing every phase of P. malariae, P. vivax rings, trophozoites and schizonts plus P. falciparum crescents=mixed B. T. plus quartan in convalescent M. T. case.

Some French workers,—chiefly on war experience in Macedonia,—have tried to make out that all three parasites are one and the same species. Base the evidence on change of type during year : in spring nearly all cases B. T. : in autumn nearly all M. T. But this is the commonplace in India, e.g. Calcutta spring cases are mostly B. T., (and many are relapses of B. T. from last year), autumn wave of M. T. cases follows. Quartan seen, but rarer, all the year round. Have not infrequently seen all three parasites in a week in the laboratory about October or November. Not unusual to get relapse autumn B. T. cases with M. T. infection super-added.

Dr. Mary Rowley Lawson believes that malarial parasites are never inside R. B. Cs., but remain throughout life cycle attached only to surface of R. B. Cs., that one parasite may, in course of life cycle, visit and destroy several R. B. Cs. Papers illustrated by beautiful microphotographs. Recently, 1922, Sinton has claimed that this view is correct. But this view is almost certainly wrong. Admit freely that malarial parasites are applied to surface of R. B. Cs., and are extracellular for part of life cycle, even perhaps for as long as 20 hours or so. But later are almost certainly inside R. B. Cs. In fact whole action of quinine depends on this fact. It destroys parasites by process of fractional destruction : parasites still on R. B. C. surface destroyed, those inside R. B. Cs. are in safe and quinine-free area and multiply undisturbed. Some authors make curious mistake of referring to gametocytes as "extracellular forms." Gametocytes are certainly not extracellular, e.g. remains of distended R. B. C. around M. T. crescent.

The student should now memorise this lecture almost by heart, and take every opportunity of studying malarial blood films and identifying the species and phases of the parasites encountered.

Literature and References.

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MORPHOLOGICAL CHARACTERS OF THE MALARIAL PARASITES.

(Romanowsky Stains) (after Christophers and others).

	"B. T. PARASITE." PLASMODIUM VIVAX.	"QUARTAN PARASITE." PLASMODIUM MALARIÆ.	"M. T. PARASITE." PLASMODIUM FALCIPARUM.
Trophozoite Early Ring.	Relatively large ; occupies $\frac{1}{4}$ to $\frac{1}{3}$ vol. of R. B. C. Round or oval. Chromatin a round dot in thin part of ring or often appears as if in vacuole.	Smaller and denser than B. T. Compact, dense, little ring, round or oval. Often seen sideways. Cytoplasm deep blue. Large deep red chromatin dot. Ring often very narrow on each side of chromatin. Text book " Signet ring. "	Smallest malarial form. Almost dots to begin with. Accole forms very characteristic. Sticks to edge of R.B.C. Multiple infection of R.B.Cs. very characteristic. Chromatin a deeply staining granule or bar. Cytoplasm very scanty.
Growing trophozoite	Flimsy. Fine streaming pseudopodia through R. B. C. Multiplicity of forms seen in same slide characteristic. Chromatin 2 or more equal round dots. Fine melanin grauules.	Compact with cytoplasm more collected together. Ribbon and comet like forms streaming across the R. B. C. rather characteristic. Melanin found early in coarse, dense, black masses and chunks.	Typical but very small ring : Occupies only $\frac{1}{6}$th to $\frac{1}{7}$th of R.B.C. Chromatin much : cytoplasm little. Chromatin often rod like and divides very early.

	"B. T. PARASITE." PLASMODIUM VIVAX.	"QUARTAN PARASITE." PLASMODIUM MALARIÆ.	"M. T. PARASITE." PLASMODIUM FALCIPARUM.
Melanin pigment	Brown black : fine grains or tiny rods.	Much. Coarse, dense rods, chunks and masses. *The* pigmented parasite.	Granular.
Infected R.B.C.	Swollen, distended, and pale. Schuffner's dots.	Not enlarged : staining unaltered.	Unchanged or even shrunken in size. Maurer's dots.
Schizont	In enlarged pale R. B. C. shewing Schüffner's dots. Flimsy. Contour irregular. Fine melanin granules. 2, 4, 8 etc. round chromatin masses.	Large round deeply pigmented body. Occupies most of unaltered R. B. C. Compact globular, clear outline. Cytoplasm stains deeply blue. 6 or 8 chromatin masses.	Not seen in peripheral blood. Small. Only occupies ½ to ⅔rds of R. B. C. 8 to 12 chromatin dots.
Sporulating Schizont : Mature rosette	Large form : 8½ μ : almost fills the distended and pale R. B. C. 15 to 20 merozoites in an irregular grape like cluster. Residual mass with melanin pigment to one side of centre.	Typical geometrically arranged "daisy" rosette. 6 to 12 merozoites symmetrically arranged round deeply pigmented, central residual mass.	Not seen in peripheral blood. Small : occupies half R. B. C. 8 to 10 merozoites arranged as in quartan. Central residual mass with fine melanin granules. Maurer's dots in R. B. C.

Young Gametocyte	Resembles growing trophozoite but has only one chromatin mass lying in a clear space more or less near the centre of the parasite. Non-amœboid.	Rarely seen. Densely globular. Very prominent large single nucleus and much coarse pigment.	Spherical: later egg shaped. Occupies only half R. B. C. Much dense cytoplasm. Star shaped single large nucleus lying in a pale area. Only seen in internal organs.
Micro-gametocyte (Male.)	Large, round body. 10 to 12 μ. Fills the distended, pale and almost invisible R. B. C. Stains badly. Cytoplasm pale grey-green or grey reddish colour. Pigment light and yellow: fine grains. Large, loosely wound nucleus, staining badly: often extends across the parasite "like a cholera belt."	Round, spherical body. Fills the R. B. C. which is not altered. Stains well. Cytoplasm deep blue. Central cluster of pigment chunks and masses. The most densely pigmented malarial form known. Chromatin loosely wound skein across the parasite and across central pigment cluster "zones of Jupiter."	Male "crescent." Sausage shaped. Shorter and stouter than female. Occupies whole R. B. C., traces of which are seen in concavity of parasite. Clear pale blue cytoplasm. Melanin as scattered fine grains in middle ⅔rds. Large, oval, central, loosely wound nucleus: stains badly lying amongst pigment grains.
Macro-Gametocyte (Female.)	Large round body, 12 to 14 μ. Fills enlarged, pale and almost invisible R. B. C. Cytoplasm stains deeply blue. Considerable granular melanin. Nuclous oval: stains deeply red: like a tightly rolled up ball of string in pale area to one side of parasite.	Very rarely seen, Resembles B. T. macrogametocyte: but is much more deeply pigmented.	Female "crescent." *Longer* than male. Pointed ends. Cytoplasm stains deeply. Central dense cluster of melanin grains. Nucleus stains intensely as compact clump in centre of parasite amid mass of pigment.
Peripheral Blood Films	Shew all phases of both schizogony and sporogony. Multiple infection of R. B. Cs. fairly common.	Shew all phases of both schizogony and sporogony. Microgametocytes rarely seen. Occasionally multiple infection of R. B. Cs.	Rings and crescents are the only forms seen in peripheral blood. Heavy multiple infection of R. B. Cs. is very characteristic: *e.g.* 2 to 8 rings per R. B. C.

CALCUTTA SCHOOL OF TROPICAL MEDICINE.

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LECTURE 12.

MALARIA, MOSQUITO CYCLE.

For a good account of mosquito anatomy, dissections etc. see Stephens and Christophers' "Practical Study of Malaria." Manson, 1894, suggested that mosquito might carry malaria : Sir R. Ross commenced work on problem, 1895; discovered first transmission cycle of Proteosoma of Indian sparrows in Culex mosquitoes. Next worked out similar malaria cycle in anopheles. Did not see actual fertilisation in mosquito midgut : this was first seen by MacCallum in similar Halteridium parasite of pigeons, and then in malarial parasites. Ross' final publication of mosquito transmission of malaria, 1898. Grassi and Bignami first gave full, detailed account of whole sexual cycle : and final proof was by experiments on human volunteers. Sambon and Low went out from London School of Tropical Medicine to infected Roman capmaigna : lived free from malaria in screened huts in heart of infected districts : fed anophelines on malaria cases, posted them to London. Here the infected mosquitoes were fed on Dr. P. Manson and Mr. G. Warren. Both had never been out of England or exposed previously to malarial infection : both took : and shewed parasites in blood. Value of human volunteer as experimental animal deserves to be noted. Mosquito malaria discovery led to whole of sleeping sickness and other insect borne protozoal investigations : and to celebrated work of Ross at Ismalia and Cyprus : and to vigorous antimalarial campaign in Panama Zone.

First few steps in sporogony cycle can be observed in shed blood without mosquito at all. Place drop of gametocyte-containing blood on clean slide, breathe on it or add trace distilled water to lake corpuscles : stages up to fertilisation can now be watched.

First change is the maturation of the gametocytes. This is a curious process akin to the shedding of polar chromatin by sex elements of metazoa. One or two small, deeply staining, tiny bits of waste chromatin are thrown first to edge of and then out of the gametocyte, in both male and female. Residual, sexual chromatin stains less deeply and becomes more or less central in position.

Maturation phenomenon first studied and most fully for the M. T. crescents by J. D. Thomson, 1917 and subsequently. M. T. crescents have a very thick cell envelope of their own, apart from investing R. B. C. membrane. When blood shed this envelope comes into prominence. In female crescent a pore or hole forms in envelope about middle of concave side : cytoplasm and chromatin flow out of envelope membrane : leaving behind empty shell and two tiny bits of polar waste chromatin. Free macro-gametocyte now becomes directly transformed into round, spherical, extracelluar, motionless macrogamete. Lies motionless in gut contents of mosquito awaiting fertilisation. In male crescent limiting membrane gives way uniformly, polar chromatin is discarded and ejected from cell, and microgametocyte rounds up and becomes extracellular.

Second step is fertilisation. Macrogamete remains as motionless, round sphere, full of food reserve material and staining deeply. In cytoplasm of microgametocyte vigorous streaming movements commence. Gradually changes to a sphere which moves jerkily about through fluid of midgut of mosquito. Suddenly 6 to 8 little protrusions of cytoplasm are shot out from the surface of sphere : nucleus divides into 2, 4, 6 or 8. Daughter nuclei travel to periphery of sphere; each is finally shot off as a microgamete with its own cytoplasmic filament. Microgametes are fine, filamentous forms, with very little cytoplasm, and mostly chromatin. At this stage the microgametocyte becomes a whirling body, and the (incorrect) term "exflagellation" is applied to this process. 6 to 8 microgametes produced from each microgametocyte.

Microgametes next scurry through fluid contents of mosquito gut in search of macrogametes. Actual attraction probably chemotaxis. Motionless macrogametes become surrounded by swarms of microgametes. One of latter enters macrogamete, leaving its cytoplasm behind : male pronucleus enters cell, unites with female pronucleus : syngamy occurs : zygote results. The steps up to fertilisation are best studied under dark ground in freshly shed blood containing gametocytes.

Zygote = travelling vermicule, becomes elongated and vermiform in shape. Progresses by a gliding, peristaltic, worm-like movement, exactly similar to gregarine movement. Often termed ookinete = motile egg. Anterior end hyaline and bluntly pointed : nucleus central : posterior part rounded and bulbous and contains most of the pigment. Posterior part is then constricted and cast off with most of pigment and good deal of cytoplasm. Ookinete travels in mosquito midgut to gut wall, penetrates between cells of epithelial mucosa, comes to rest in the tunica elastico-muscularis, just between inside gut epithelium and outside muscular wall of stomach. Now rounds up, secretes thin cyst wall, becomes motionless and oocyst. The oocysts are found especially on the posterior part of the midgut : at 12 to 24 hours after feeding mosquito's gut is full of ookinetes : oocysts form 3 to 4 days after feed.

Oocyst next grows very rapidly in size. Stomachs of infected mosquitoes very pretty picture : see Stephens and Christophers' for plates. On focussing down on dissected midgut we see tracheæ, muscular and elastic fibres, cells of gut epithelium, brown pericardial cells, cells of the fat body, crystals, etc., sometimes natural herpetomonad of the mosquito. On focussing down oocysts come into view, first hazy in outline, then suddenly in clear, optical section. Appearance of pigment in them often the first indication of their character. Usually find anything from 6 to 30 oocysts at all different stages of development in same specimen. As many as 500 have been reported in a heavily infected mosquito.

Young oocyst at about 40 hours from feed is about 12 to 14 μ diameter. Nucleus now divides. Usually 8 main daughter nuclei are produced. Around each gathers cytoplasm. There are thus produced 8 sporoblasts, but these are curious IN THAT THEY HAVE NO INVESTING MEMBRANE, and are naked. Grassi applied term sporoblastoids to them to emphasise this character. They are separated from one another by clefts and cracks in the cytoplasm of the oocyst, and connected by fine thread-like strands of cytoplasm. In addition to them is small mass of waste residual cytoplasm=nucleus de reliquat.

Nucleus of each sporoblastoid next divides into very numerous daughter nuclei, and these, AS IN THE COCCIDIA, pass to the periphery of the sphere and are arranged around the surface of the naked, non-invested sporoblastoid. Around each cytoplasm aggregates, a protrusion is put forth from the surface, and these grow out vertically from the surface of the sporoblastoid, invested in thin cell membrane and each becomes a sporozoite.

Thus ripe malarial oocyst or pansporoblast in gut wall of mosquito has—1. thick refractile cyst wall of its own ; 2. inside it are 8 or more badly defined and naked 'sporoblastoids.'

3. Each sporoblastoid is producing hundreds of sporozoites. In consequence the hundreds of little sickle shaped sporozoites lie in parallel rows, like faggots in a bundle : and on focussing down into optical section of a ripe oocyst we get the appearance of myriads of fine parallel striae crossing each other in all directions=bundles of unstained sporozoites. Finally 4. we have the waste residual nucleus de reliquat. The mature ripe pansporoblast is about 50 to 60 μ in diameter and may contain from some hundreds to 10,000 sporozoites. The oocysts are ripe at about the 8th day after feed, but see later.

The ripe cyst now bursts, not back into the gut, but outwards into the cœlomic body cavity of the mosquito, setting free myriads of sporozoites. Malarial sporozoites are most unmistakable objects, about 10 μ long, by 1 to 2 μ broad. Sickle shaped and sharply pointed at each end. Cytoplasm stains a pale Cambridge blue : nucleus is at centre, oval or round, stains deeply ; no pigment. Move :—1. by forward progression in own longitudinal axis ; 2. bend tip to tail and then straighten ; 3. snaky, bending movement slow, dance-like in character. Movements are slow, unhesitating and continuous. Never jumpy and are most deliberate : nothing else in protozoology quite resembles this : movement quite characteristic.

Liberated sporozoites wander through and penetrate into EVERY tissue of the mosquito. Have been recently found, Muhlens, 1921 in sections of thorax muscles, wings, neck, head, legs, heart, aorta, scutellum etc., but NOT in the ovaries. But the blood currents gradually drive them into one SPECIAL place, the posterior end of the trefoil shaped, lobed salivary glands ? ? does chemotaxis play a part. Swarming around the rounded, blunt posterior ends of the salivary glands they gradually wriggle their way through between the cells and accumulate in myriads in the acini of the salivary glands. By about the 10th to 11th day after feed the mosquito's glands are packed cram full with sporozoites.

When such an infected mosquito bites a healthy person a large dose of sporozoites is injected down the salivary ducts into the patient. The injected sporozoites pass into the patient's blood and make for the R. B. Cs. They enter into the R. B. Cs., become malarial trophozoite rings, and commence a new schizogony cycle. Thus second individual infected. Theatrical picture by Schaudinn of a malarial sporozoite entering an R. B. C. almost certainly NOT drawn from life. Says the process of entry takes 40 minutes.

Biological Factors affecting the Malaria Mosquito Cycle. These are MOST important. Oxygen is essential: male gametocyte does not "ex-flagellate' unless air or oxygen available. 2. The actual liberation of the gametocytes from the R. B. Cs. is probably an osmosis effect, due to sudden alteration in the density of the blood after it has been ingested by the mosquito: and imitated on the slide by adding water. 3. A lowered temperature is absolutely essential. Thus for the B. T. parasite sporogony cycle goes through in from 8 to 9 days at an optimum temperature of 77 to 86°F: (25 to 30°C): at 24°C it takes 10 to 12 days, between 18 and 22°C it takes 18 to 19 days, below 16°C development ceases or becomes abnormal: above 35°C, 95°F, development also ceases or becomes abnormal. Finally there must be present some other special factor which determines why some anopheline species carry malaria, whilst others do not: a factor as yet of unknown character. King and Hodgson emphasise that it is not so much dry bulb as wet bulb readings that control the rate of development.

How long does infected mosquito retain infection? Point not yet settled. Roubaud found that infected mosquito lost most of its sporozoites at first few bites: others have found infection retained 45 days. After hibernation, several workers claim that infected mosquito becomes non-infective.

On other hand Wenyon, 1920, in Macedonia found that mosquitoes DO retain infectivity after hibernating. Exposure up to 12 hours in ice chest does not prevent development of oocyst. After three weeks' exposure to winter temperatures when mosquitoes warmed, sporozoites developed. Question of infectivity in mosquitoes hibernating over winter still not settled. With regard to epidemiology what one gets in India (and Macedonia) is (a) spring wave of B. T.=relapses from last year's cases with fresh infection of mosquitoes: followed by (b) rise of M. T. wave in autumn, (accompanied if heavy rains plus high food prices by epidemic state): whilst (c) quartan is more or less at a constant level the year round. It is this fact which has misled French workers who believe that all three species are the same, and that M. T. is a seasonal variety of B. T.

Possibility that infected mosquito may transmit to next generation: so far no evidence of it. Muhlens on section cutting failed to find infection of ova. Cycle (which is quite unlike that of piroplasma) is against it.

On other hand transmission from mother to fœtus almost certainly occurs, and is a point worth attention.

After sporozoite has been injected there follows the latent period of incubation, during which parasites are multiplying but still not sufficiently numerous to cause symptoms, (Ross and J. D. Thomson put the dose of parasites necessary to produce febrile symptoms at 250 million in the body). Latent period of course varies with dose of sporozoites injected : thus as Christophers has well shewn a single but heavily infected mosquito may do far more damage than dozens of lightly infected ones. Approximate incubation periods are 14 to 18 days in B. T., 18 to 21 days in quartan, 9 to 12 days in M. T.

Points relating to differences in species. At optimum temperatures, about 25°C, B. T. sexual sporogony cycle takes 8 to 9 days, quartan about 18, M. T. 9 to 10 days. Sporogony cycle of malaria was first worked out by Ross for B. T. in Anopheles claviger.

B. T. oocysts have a dull, feebly refringent look in the fresh state :—pigment present in slender chains or wisps, pale yellow in colour and not very distinct. Quartan oocysts shew dense, black pigment in a coarse clump. M. T. oocysts look like glass globes : have sharp, well defined contour, and are highly refringent. The pigment is pepper-like, consisting of black grains. M. T. will develop at a slightly lower temperature than B. T.

Four great malaria transmitting anopheles of India are :—(a). A. culicifacies : 4 % sporozoite rate in Mian Mir epidemic (Christophers) : 8 % in Ennur outbreak. (b). A. listoni, variety funestus, 6 % sporozoite rate in Punjab. (c). A. stephensi. 3 to 10 % sporozoite rate in different parts of Bombay. (d). A. fuliginosus, sporozoite rate in nature 1 per 200 infected. Other Indian carriers are (e). A. maculatus, especially in the Deccan foot hills and Bombay presidency, and in Shillong. Also A. ludlowi, A. minimus and A. willmori (this last is the mosquito of hill station malaria, and is a tree breeder). A. rossi is a problem : can be easily infected in the laboratory, and is the commonest Bengal anopheline, but never found infected in nature. Christophers dissected 496 in Mian Mir epidemic and 364 in Ennur outbreak, but found none infected. Statement often made that A. punctipennis carries, but Mitzmain failed completely in most careful experiments to experimentally infect it in the laboratory. Where a mosquito carries M. T., it will also carry B. T. and quartan : there seems to be no exception to this rule.

In Mesopotamia A. stephensi the chief carrier, with sinensis, maculipennis, and pulcherrimus also. In Palestine maculipennis chiefly. In Africa the great malarial mosquito of the

West Coast is A. or P. costalis : also A. funestus and albimana in tropical Africa. In Europe A. bifurcatus, A. maculipennis, and A. quadrimaculatus proved carriers : with A. plumbeus experimentally infected in the laboratory and suspected. Excellent account in James' "' Malaria at Home and Abroad " of Sheerness outbreak in Thames estuary from returned soldier convalescents : maculipennis was the carrier. Amusing incident in the war of Germans infected in Alsace from French Algerian troops in opposite trenches full of malaria, with A. plumbeus probable carrier.

Mosquitoes often have natural protozoal parasites of their own, e.g. herpetomonads very common. Ross discovered a Nosema parasitic in Anopheles : with black spores : and it infected not only the mosquito but also the malarial oocysts in the mosquito : a splendid instance of hyper-parasitism.

Darling proved experimentally that a mosquito does not take the infection from the human host unless there are at least 12 gametocytes per c.mm. of blood. Usually believed that if patient is on full quinine his gametocytes will not develop in the mosquito, but this is doubtful, and also doubtful whether quinine affects gametocytes at all. In malaria cases on full quinine one may sometimes see affected rings and growing trophozoties looking shrunken, distorted, and badly staining : but the gametocytes seem unaffected and stain normally. One reason for the special epidemicity of the M. T. parasite is supposed to be that it produces a larger percentage of gametocytes than do the B. T. and quartan : and also the M. T. gametocytes persist far longer in the peripheral blood then do the B. T. and quartan gametocytes. On other hand quartan infection persists in the patient longer than do B. T. and M. T.

Note that the finding of M. T. crescents in blood films from persons apparently in good health and with no recent fever is quite common.

Malarial Relapse. The History of a Mistake. Schaudinn, 1902, had under treatment a Jewess at Hamburg suffering from B. T. chronic malaria. Just prior to a relapse he took blood films and in them found forms which he believed to be macrogametocytes breaking up by parthenogenesis into merozoites. On the strength of this one case and one set of films Schaudinn assumed that relapse in malaria is due to (a) gametocytes are very resistant forms which he thought lived for weeks or months undisturbed in peripheral blood. Next, said he, if patient now gets a chill or gets run down these macrogametocytes break up into merozoites, schizogony cycle sets in again and fever recurs = relapse.

Schaudinn so dominated protozoology that this was at once and generally accepted. Hartmann even went one better, and in H. muscae domesticae described male and female herpetomonads and etheogenesis, i.e. production of young by a male parent, with no female concerned, as occurring. For 20 years Schaudinn's views unquestioningly accepted and dominated protozoology.

Now parthenogenesis, i.e. formation of young by an unfertilised female parent, or virgin birth, is a quite common and well known phenomenon among insects, and has been experimentally produced in frog's eggs by Loeb. No reason at all why it should not occur among the Protozoa. Yet anyone with any knowledge of protozoology can see that Schaudinn's view must, on the face of it, be wrong. A parthenogenetic macrogametocyte would not produce merozoites at all (and if it did the process would not be parthenogenesis). It should produce sporozoites via an oocyst phase. Hence on the purely protozoological point of view Schaudinn must have been wrong.

Schaudinn's figures were explained in 1917 by J. D. Thomson. This knocks the whole bottom out of Schaudinn's views. Thomson had opportunities at home hospital for convalescent malarial soldiers from war areas of examining hundreds of B. T. relapse cases.

A ring parasite inside an R. B. C. may develop into either (a) a schizont, or (b) a gametocyte. Now suppose two rings get into the same R. B. C. When they grow up we then have such combinations as schizont plus schizont, or gametocyte plus gametocyte, or schizont plus gametocyte. Schaudinn's figures were schizont plus gametocyte in the same R. B. C., and if one only focusses carefully one always sees the dividing line between them. Every subsequent worker has confirmed Thomson's observations, and one may easily find such forms in examining slides.

Parthenogenesis may occur among the protozoa, but its existence has not been proved, and it is not the explanation of malarial relapse.

Spontaneous Cure and Relapse in Malaria. Suppose the B. T. schizont gives birth to 20 merozoites. Actual true figure is nearer 14 : but 20 is a convenient figure to work with. Suppose a mosquito injects into a person only one sporozoite and that its multiplication goes on undisturbed : the progeny of one sporozoite would be 512 millions on the 18th day = fever.

If uninterrupted should be 204,800 millions on 22nd day, and patient should be dead. As patient does not die, however, and as tendency even in untreated malaria is towards spontaneous (even if temporary) cure, there must be a terrific destruction of parasites. This takes place especially in the spleen, which is the great site of parasite destruction. Blood from the splenic vein in malaria is very rich in haemozoin.

Process of destruction of parasites is as follows :—Only those which are still attached to the surface of R. B. Cs. are affected. Those inside the R. B. Cs. are safe and free from any external disturbance and multiply unaffected. The attached parasites are swept off in thousands, swept in the circulation into the spleen meshes, and there caught. They then simply go to pieces. The chromatin breaks up into fragments =chromorrhexis and is dissolved = chromatolysis. Finally only cytoplasm and pigment are left. The cytoplasm becomes very swollen and vacuolated and dissolved = cytolysis. Finally only free pigment is left, and this is mopped up by the large hyaline mononuclears and macrophages and wandering plasma cells and endothelial cells = macrophages. One may often come across tiny sheets of cytoplasm plus pigment only, blocking the intercellular spaces.

Contrary to usually held opinion there seems to be NO phagocytosis concerned, and the parasites are NOT engulfed at all : only their residual pigment.

Unlike kala azar, spleen puncture in malaria is of little or no value for diagnosis. Occasionally one comes across a crescent in spleen puncture films where they would be missed in the peripheral blood. Often one comes across undoubted hemaozoin pigment : pigmented or more accurately pigment-bearing large mononuclears are often seen in spleen films : but on the whole spleen puncture is of no value in malarial diagnosis. Following the general protozoal rule the gametocytes make especially for the peripheral blood rather than for the internal blood stream in order to gain access to the mosquito.

This, then is one of the most important factors in spontaneous cure, destruction in the spleen of parasites which have not got a firm hold of the R. B. Cs. A second factor probably is a gradual change in the permeability of the R. B. C. envelope, which by degrees becomes more resistant and which the parasites are unable to penetrate.

A third, and exceedingly important factor is gametocyte production. Every ring which grows into a gametocyte instead of a schizont represents one non-multiplying form introduced into the multiplication rate: and thus removed. Thus take above figure of 512 millions on the 18th day infection, suppose 90% of parasites per 48 hours are destroyed or become gametocytes : at next 48 hours 51 million left : their progeny in another 48 hours becomes 1020 million = febrile dose = disease progressing. If destruction rate be 98% however, of 512 millions, 502 million are destroyed and only 10 million left; their progeny in 48 hours = 200 million = dose not sufficient to cause fever = spontaneous cure.

In other words we have a febrile threshold for malaria. placed at a total dose of 250 million parasites by Ross and Thomson. If destruction plus gametocyte formation = 98% rate, spontaneous and absolute cure occurs. If only 95% disease is progressive. But at a combined figure between 95 and 96% we reach a condition of balanced equilibrium : parasites are multiplying by schizogony, parasites are being destroyed, and are being converted into non-multiplying gametocytes : the product is always below 250 million parasites, i.e. below the febrile threshold : patient is in a latent state. Relapse occurs if either the threshold be lowered or the patient's health be so disturbed from extraneous causes that the destruction rate is lowered. There is no necessity at all to postulate either (a) safe areas in the body which parasites get into and are protected from destructive agencies : (the only really safe area for them is inside the R. B. C.) : or (b) special and as yet undiscovered forms of parasites, especially resistant and associated with relapse. Relapse is a question of mathematics and the threshold.

Miscellanea. Mesnil and Roubaud, 1917, succeeded in infecting two chimpanzees with the B. T. parasite from man, after innumerable previous failures. Both monkeys received big doses of blood laden with B. T. schizonts intravenously. Neither shewed fever, but both shewed scanty parasites multiplying in blood for a few days only. The natural plasmodia of apes have nothing whatever to do with human malaria and the human malarial parasites are strictly specific to man, and to certain anopheline mosquitoes only. Why ? ?

Knowles, 1919, (before seeing Thomson's paper) thought " If Schaudinn right and relapse in malaria is due to parthenogenesis, let's get a convalescent M. T. case whose blood day by day shews nothing but numerous crescents : and no schizogony forms. Let's get an immense dose of crescents from such a case : inject intravenously into an anthoropoid ape, and see what happens. If Schaudinn right and ape susceptible, we may perhaps get true parthenogenesis, and be able to study the process in hourly blood films." Did so accordingly : using a case with 250 million crescents per c.c., and a hanuman ape (the small anthropoid of Assam). The intravenous injection proved very difficult : femoral vein had to be exposed by incision, and there was much handling and shock. Second day nothing found in films from monkey except obviously degenerating crescents. Third day monkey suddenly became acutely ill with subnormal temperature and collapse. Films shewed an intense infection with plasmodial forms : but on careful examination it was clear these had nothing to do with the human parasites injected : but were those of a plasmodium natural to the monkey itself : Plasmodium semnopitheci. This parasite seems about intermediate in morphology between human B. T. and quartan. Monkey died evening of the third day from the infection. Unfortunately its blood was not examined prior to the experiment. Death due to lighting up of natural plasmodium infection from shock of operation.

" Malaria toxins." There is little or no evidence that the malarial parasites produce toxins. Views as to the production of a haemolysin, and endolysin and a pyretic toxin produced by parasite probably wrong. Blood from patient filtered free of parasites causes fever on injection into healthy person, but no parasites and no recurrence. Recent work of great importance by French workers on the malarial rigor as an anaphylactic phenomenon, or " haemoclastic crisis." When rosettes rupture into blood the merozoites probably act as foreign proteid, and the rigor=proteid shock, like that e.g. produced by intravenous T. A. B. vaccine. Other workers hold that parasite does produce a definite haemolysin, since many more R. B. Cs. are destroyed than the mere presence of parasites will account for. Quite possibly the disintegrated remains of the ruptured R. B. C. may act as foreign proteid. Much biochemical work on this subject badly wanted.

Three interesting cases reported during the war where in doing blood transfusions a malarial donor was used by accident, and the parasites were accidentally injected into the recipient, who developed malaria with parasites in blood films : accidental mechanical transference.

The importance in India of childbirth in waking up a latent malarial infection is not half realised today. Malaria is certainly acquired congenitally. Heiser had a case in which an infant

7 days old had fever with M. T. crescents in its blood. As crescents only form from 8th day of infection onwards this infant must have been infected in utero.

There is no real immunity to malaria. In fact it is very doubtful whether man acquires immunity to *any* protozoal infections. What he acquires is not immunity but tolerance. Patient gradually gets accustomed to his parasites which also by degrees die off. The whole question of the so-called immunity in protozoal diseases is very dubious. Thus Dobell gives instances of E. histolytica infection tolerated for years without symptoms : T. lewisi of rats is harmless to them ; E. coli may perhaps have been a pathogenic protozoon several million years ago, but man has now got accustomed to it ? ?

Superinfection has been shewn to be possible in malaria : i.e. a person suffering from malaria may get a fresh dose from another infected mosquito. Christophers has shewn that with Proteosoma of sparrows, birds already infected can be given extra superinfection by feeding infected Culex on them. The Sergent brothers have recently shewn that blood taken from sparrows with old Proteosoma infections, where parasites cannot be found in blood films, is still infective to fresh birds intravenously.? ? this probably merely means that parasites are so scanty that they are not found.

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LECTURE 13.

Laboratory Examinations in connection with Malaria. Blackwater Fever.

The following procedures may be required :—

1. Examination of fresh unstained blood, but more open to errors than that of stained films. Rings have opaque white look; B. T. shews amoeboid activity with haemozoin being carried about the R. B. C.; quartan pigment very striking; crescents easily seen. To count crescents adopt exactly same technique as for total leucocyte count.

2. Examination of thin well stained films. James says if only one slide be examined positive results in only 40% cases. 4 to 6 films on 4 to 6 occasions better. Christophers says dont give a negative finding till 100 microscope fields searched. Previous quinine administration renders search for parasites hopeless. Early M. T. infections with very tiny rings those most frequently overlooked. Dont diagnose anything as malarial parasite unless 1. it focusses in and not upon the R.B.C. 2. has blue cytoplasm. 3. definite outline. and 4. red chromatin. Anything doubtful is probably not a parasite.

3. Thick film method. Take a perfectly clean slide and flame it. With sterilised big platinum loop take four big drops of blood and place them at corners of a small half inch square on centre of slide. Pool into a uniform thick film either with a round needle or platinum loop. Essential to avoid gross bacterial contamination which much interferes with examination of the film. Allow to dry throughly, protecting from dust and flies by Petri dish inverted over it. Drying takes at least half hour, often more. When thoroughly dry either :—(a). place slide standing vertically in bowl of distilled water till all Hb. dissolved out, 20 minutes. Then remove very gently as film is not fixed and stain by Leishman's stain. Or :—(b). Ruger's method. Move the slide gently about in a solution 1% acetic acid, 2% formalin. After laking is complete treat in same way in tap water to remove acid etc. Wash gently in distilled water and stain by dilute Giemsa.

In thick films crescents and gametocytes shew up well. The R. B. Cs. however are laked and the method is only of use to supplement, not to replace the examination of thin films. As a rule in a case of suspected malaria take three or four good thin films, with even leucocyte edge in case a differential leucocyte count is subsequently desired, and one thick film.

4. The finding of pigment-bearing macrophages in stained films is diagnostic of malaria. These are either large hyaline mononuclears or endothelial cells which have phagocytosed the haemozoin pigment set free on rupture of the rosettes. But be certain that it is true haemozoin pigment, and neither dust, (which is present both on and outside the cytoplasm of the cells), nor the very common extruded granules of chromatin frequently met with in mononuclear leucocytes in tropics, and which stain purple. True haemozoin is brown-black. Spleen puncture smears in malaria often shew such cells very well.

5. Spleen puncture of little value in diagnosis of malaria, Acton and Knowles, 1923. Degenerated parasites and haemozoin are the chief positive findings and the former are hard to identify.

6. The total leucocyte count sometimes of value. For details see Stitt or other text book. Dilute the blood 1 in 20 with one per cent. acetic acid. If the ruled lines on the hæmacytometer are very faint they can be rendered distinct by very lightly rubbing a soft pencil over them and then dusting off all excess, but this is NOT recommended as it may obscure details. Always filter the acetic solution before use. Never be content with less than three counts, each of the total square ruled area of the haemacytometer. For Thoma Zeiss instrument, with 1 in 20 dilution and three such total counts = n leucocytes seen : then total leucocytes per c.mm. of undiluted blood $= \frac{n}{3} \times 200$. An Ehrlich square ocular eyepiece is extremely useful in doing such counts. In malaria one gets (a) leucopaenia, even down to 2,000 per c.mm. during the febrile phase : with (b) leucocytosis of mild grade during afebrile interval, the total leucocyte and temperature charts being the inverse of one another. The first and second rigor in an M. T. infection however may shew leucocytosis in place of leucopaenia.

7. The differential leucocyte count. Here some difference of opinion as to best procedure. Usual procedure is to count along leucocyte edge of film, but as shewn by Napier, 1922, this quite unreliable. Napier advises either (a) take an exceed-

ingly minute drop of blood, no larger than a pin point, spread very tiny film and record every leucocyte in it. Or (b) count horizontally in lines across the mid length of an ordinary film. Never be content with less than 250 leucocytes, any smaller figure quite unreliable. Easiest method is to record on ruled paper with 250 squares space outlined, so that one gets to the 250 mechanically. Or if ruled paper not available then may proceed as follows :—Let P=polymorph., M=mast cell, E= C. G. Eosinophile, L=lymphocyte and small mononuclear, and H=large hyaline and transitional : count the Ps. mentally and record in fifties, whilst setting down on paper the others. Thus an actual count read :—

P 50+50+50+30

LLHLLLEHHHLHHLHLLHLEELLLLHHLLLHLL

HHHHLLLLHLHEHHLELLEHHLHLLLHHHMLLHPHH

in a chronic B. T. case. By the time one has counted 100 Ps. the total of leucocytes counted will be in the neighbourhood of 200, and one then counts up and carries the count to a total of 250 leucocytes. Now add up : e.g. above : M 1 : E 6 : L 36 : H 31=total 74 : which subtracted from 250 leaves P=176 without the trouble of counting them separately. Next multiply by 0.4 to get percentage, which here gives :—Mast cells 0.4% : C. G. Eosinophiles 2.4% : Polymorphs. 70.4% : Lymphocytes and small monos. 14.4% : Large hyalines and transitionals 12.4%.

As a rule in malaria there is a considerable increase in the large mononuclears, e.g. 10 to 16% readings. According to Christophers a figure of over 15% (in the absence of kala azar) is proof of malaria, whilst if 20% be encountered further search of the film will usually shew a parasite. J. D. Thomson on most careful examination of whole question of leucocytes in malaria concludes :—(a). fever plus leucocytosis plus increased percentage of polymorphs. is not malaria : it may be sepsis. (b). fever with leucopaenia and a high large hyaline mononuclear percentage, e.g. 12 to 15%, is strong confirmatory evidence of malaria, (in absence of kala azar). (c). persistently high large mononuclear percentage with from time to time a leucocytosis should arouse suspicion of malaria. Leucocytosis *per se* does NOT exclude malaria.

8. Examination of the complete blood picture may be of subsidiary value, Hb. (best taken by Tallquist's blotter book) : total R. B. Cs., etc. Sp. gr. of blood slightly reduced, and R. B. C. count down to say 3½ to 3¼ million. In doing the total R. B. C. count dilute the blood 1 in 200 with normal saline : count 100 small squares, i.e. five lines of 20 sqq. each,

and the total number multiplied by 800 will give total R. B.Cs. per c.mm. of blood. Remember cardinal fact that small square of a Thoma Zeiss haemacytometer has diameter 1-20th of a mm., and volume over it is 1-4000th of a cubic mm.

9. CULTURE IS ONE OF THE MOST SUCCESSFUL METHODS OF BLOOD EXAMINATION in difficult cases, with scanty parasites. Introduced by Bass, 1911. Thomson's modification of Bass's technique best. Into dry, sterile test tube place 1-10th c.c. of 50% sterile Merck's dextrose solution. Draw 10 c.c. of blood from patient's vein : and place it in the test tube. With sterile wire rod stir gently, avoiding air bubbles, to defibrinate. Pour the defibrinated blood to which the glucose has now been added into a series of small sterile test tubes, so that one inch column in each. Insert the cotton wool stoppers, cover with rubber caps, and incubate at 40° to 41°C. The fluid divides into three layers : above plasma : below R. B. Cs. : between thin layer, about 1-20th inch, in which leucocytes plus R. B. Cs. Growth occurs only in this intermediate layer. From time to time remove samples of the intermediate layer with fine sterile capillary pipette, smear blood films, stain and examine. To make sub-cultures use same technique, using blood of healthy non-malarial donor, and inoculate by injecting from capillary pipette infected R. B. Cs. taken from the middle layer. Sinton, 1922, has most ingenious modified Wright's capillary tube in which whole process can be carried out from finger blood, but making of apparatus a little difficult. Recommends culture at 35° to 38°C. Stitt states that P. falciparum in culture shews 32 merozoites as against usually 10 to 12 in body ? ?

10. Animal inoculation useless in malaria. Parasites strictly specific to man. Mesnil and Roubaud, 1917, succeeded in transmitting B. T. infection to two chimpanzees, but infections were transient, afebrile and merely multiplication for a few days only.

11. Urine examination of subsidiary value. N excretion increased. Also especially increased is urobilinogen excretion. Schlesinger's test for it :—To 5 c.c. unfiltered urine add 5 c.c. saturated alcoholic solution of zinc acetate. Shake. Add 5 to 8 drops of Gram's iodine and shake. Filter through dry filter paper into long, narrow test tube. Hold tube containing filtrate vertically over black blackground and shake. If urobilinogen in urine filtrate shews a definite green fluorescence. Acton and Knowles found this reaction positive in 18% of persons who denied all history of fever, but positive in 58% of those who admitted to malaria within previous three months. But is even more frequent in kala azar. Urobilinogen in urine

derived from broken down Hb. in body. In malaria more usually urobilinogen but no albumin : in k.a. often associated with albuminuria.

12. Even chemical examination of stools in malaria may be of interest. Iron and urobilinogen content much increased from Hb. break down.

Malaria. Miscellanea. The rigor in malaria is almost certainly of the nature of an anaphylactic shock. Older views as to formation by parasites of toxins, etc. probably quite wrong. Doubtful indeed, to what extent protozoa form toxins at all : they act more by tissue destruction. When the rosettes rupture either the merozoites, or—more probably—the proteids of the destroyed R.B.Cs. thrown into general circulation and cause proteid shock, the "haemoclastic crisis". Symptoms of malarial rigor=those of proteid shock as induced by intravenous injection of foreign proteid : lowered arterial tension, leucopaenia, lowered R. B. C. count, altered differential leucocyte count. Abrami and Senevet shew this and that injection of adrenalin 1 to 2 hours before anticipated rigor will often abort it : saved 4 out of 6 very desperate cases with it.

On rupture of rosettes haemozoin picked up by macrophages and carried to internal viscera as spleen and liver, whence their pigmentation in chronic malaria. Malarial anaemia due to (a) destruction of R. B. Cs. by parasites : (b). destruction of other R. B. Cs. in the rigors by haemoclastic shock, (? ? due to formation of haemolysin by parasites ?) : (c) the capillary haemorrhages associated with the disease. Products of destroyed Hb. dealt with in liver : end results urobilinogen. Note frequency of hepatic disturbance in malaria. Some of destroyed Hb. laid down in liver and spleen as haemosiderin, which gives Prussian blue reaction with KCN and HCl.

Much dispute as to length of life of gametocytes. Some hold view that they live for weeks and months in peripheral circulation. Have met with a case at Shillong where nothing but crescents seen in daily blood films for three weeks. Recent careful study by J. D. Thomson says life of crescent does not exceed about 10 days, that of B. T. gametocytes probably not much more than 5 days.

Best account of malarial epidemiology probably that in James' "Malaria at Home and Abroad." The economic factor in malaria of the utmost importance as emphasised by Christophers, Bentley and others. Thus if we get following events :—(a) one or two years of famine or very high food prices : followed (b) by heavy monsoon with abundance of anopheline carriers : then (a) with lowered resistance of people the number of malarial carriers with gametocytes in peripheral blood will be excessive and the resistance of population in general low : (b) the cattle die off, leaving a smaller feeding ground for anopheles, which will therefore concentrate more on man : (c) and hyper-epidemic fulminant malaria sets in, always of M. T. type, with 80 to 90% of populations infected, incubation rates of only a few days, and terrific and most unusual mortality, as in great Punjab epidemic of 1908. See also most interesting paper by Gill, 1923, on the forecasting of malarial outbreaks by paying attention to such factors.

Malaria-Mosquito Work.

1. Dry test tubes, dry cotton wool, dry wide mouthed bottle. Search cattle sheds and under eaves of houses especially, for anophelines. Capture in test tubes, transfer to bottle and bring to laboratory.

2. Keep mosquitoes in chutney jar. Insert bit of cardboard across inside for them to rest upon. Turn jar upside down. Remove hollow stopper and fill it with water, on which float bit of white cardboard on cork : invert jar into it, after putting mosquitoes inside. Next morning males are all dead, and ova all laid on paper. Collect latter to breed out.

3. On 2nd or 3rd day must feed mosquitoes. Remove stopper of jar. Cover with mosquito netting tied over mouth. Rest arm on it at night : mosquitoes come up and feed. Or can be fed on shaved abdomen of rabbit. Be certain that donor has no natural protozoa in his or its blood.

4. To breed out clean insects, take four square frame of bamboo : cover with mosquito netting. Inside place saucer of water with ova in it, and hang up bananas inside for mosquitoes to feed on. When they hatch out they fly to the netting and can there be caught.

5. In making experimental laboratory feeds use only clean laboratory bred insects. Dissect a sample of them to make certain free from natural protozoa of their own. Feed as above, but on malarial carrier with gametocytes in blood. For Culex work with Protosoma use box with muslin sleeve projecting from its open end. Tie cloth round bird's head and tie feet together to prevent its struggling. Put into box : insert mosquitoes : pull sleeve tight.

6. Recently fed mosquitoes shew red distended mass in abdomen. In females when gravid, ovaries visible as two whitish lumps one on each side of hinder part of abdomen. Select for dissection only females which have had full blood meal.

7. Dissection : kill by tobacco smoke or chloroform vapour. Brush off scales. Remove wings and cut off legs. Place on slide under dissecting microscope in 0.5 % saline. Use finely ground down needles ; old cataract knives re-sharpened useful. With left hand needle fix thorax. With right hand needle make transverse nick at each side of abdomen between 6th and 7th segments. Loosen last segment from abdomen. Separate needles in two hands by slow, gentle, steady traction. Stop before gut ruptures. Mid gut and hind gut with attached organs come out. Cut away from thorax and transfer to clean slide and saline. Next cut away Malpighian tubules : get mid gut only clean : transfer to fresh slide and saline. Put on cover slip over intact gut and squeeze gently. Examine under ordinary microscope and 1-6th objective. Note structures seen : tracheae, muscular and elastic fibres, stellate tracheal cells, pericardial cells, fat cells. Haemozoin pigment usually first sign of malarial infection : then oocysts come into view in optical section.

8. To make preparation permanent, keeping object under microscope, place successive drops of either 2 % formalin or Schaudinn's fluid at one side of cover slip, and with blotting paper bits draw fluid through for about 20 minutes till whole is quite opaque. Gently remove cover slip from slide : gut adheres to one or other, usually to cover slip. Now treat cover slip or slide with attached gut as if it were a section, stain by any suitable process, Haedenheim's best, mount in neutral balsam.

9. Salivary glands lie in thorax, ventrally, just above origin of first pair of legs. With whole mosquito available place in saline under dissecting microscope on right side. Steady thorax with left hand needle. With right hand needle on back of head carefully and gently pull head away from thorax : use dark background. As head is pulled away from thorax glands come into view as glistening, pearly white, trefoil shaped bodies. Steady head with left hand needle and with right hand needle separate glands from head. Transfer to clean slide and saline.

If gut has previously been removed procedure more difficult. Three cuts will isolate the glands. (a). oblique antero-posterior one to sever main mass of thoracic muscles. (b). second at right angles to first behind attachment of first pair of legs. (c). third across neck parallel to first. Glands lie in mass of muscle thus isolated and must be teased out.

10. Having got glands out clean on one or both sides transfer to clean slide and saline. Put on cover slip, squeeze gently,

examine under 1-6th objective. To make specimen permanent blot off excess of saline with bits of filter paper at edge of cover slip : squeeze : draw cover slip along slide so as to make a smear. Either wave in air till both cover slip and slide dry, and fix and stain both with Leishman's stain and mount, after dehydration, in cedar wood oil : or treat as section, fix with Schaudinn's fluid, stain by Haedenheim's iron haematoxylin method.

The spermatheca can be teased out from the 6th abdominal segment, =reserve organ for storing spermatozoa in the female till wanted. The ovaries are brightly white, pyriform in shape and in last few abdominal segments of abdomen : they appear when the gut is drawn out in dissecting.

Serial section cutting of insects always a much better way of demonstrating protozoal infections than dissection : preserves relationships of parts. Procedure however tedious and troublesome. For details see Patton and Cragg's " Text Book of Medical Entomology." The last chapter in this book should be read by everyone who has much of such work to do. To fix a mosquito or other insect for section cutting, make tiny incision on each side of abdomen to let in fluid. Drop into hot Schaudinn's fluid at 60°C : leave 15 minutes. Then bring fluid just to the boil, to expel air from insect tissues, and leave three hours or more in cold to harden. Transfer to 70% alcohol next day.

Note that absolute alcohol is never absolute in India. To make it really absolute following procedure is necessary. Powder some green copper sulphate crystals. Place in big evaporating basin. Heat with constant stirring with glass rod till reduced to anhydrous, quite white powder. Allow to get cold. To bottle of absolute alcohol add one third its volume of the desiccated copper sulphate powder. This removes the last traces of water from the alcohol and makes it truly absolute. If the copper sulphate turns green it must be replaced by fresh.

Malarial Surveys.

Suppose, e.g. that " Dustypore" has acquired an unenviable reputation for malaria, and you are sent to investigate reasons.

1. Collect all available statistical information and meteorological figures for past years and months with hospital and school statistics etc.

2. Spleen index=percentage of children between ages of 1 and 10 with palpable spleens. Determine by examination of school children. Whilst doing so also take a blood film from each : (being careful to label with name and date and to put into box away from flies). Parasite index or endemic index =percentage of such children with malarial parasites in films. Gametocyte index=percentage of such children shewing gametocytes in films=malarial carrier index. This constitutes the human reservoir of the infection in Dustypore.

3. Carry out control observations on adults, taking similar observations. Where malaria is endemic we get high splenic and endemic index in children, but not in adults. Where malaria is of recent importation in epidemic form adults and children equally affected. A high percentage of infection in adults resident in the district means recent importation from outside.

4. Make a detailed spot map of Dustypore shewing all known human malarial carriers and where each lives. We have now ascertained the human aspect of the problem.

5. Examine sample catches of all local anophelines. Make a detailed list of local species. Make a spot map shewing what known carrying species are prevalent, where and when breeding, and in what areas most prevalent.

6. We can now say (a). where the human reservoir underlying the epidemic is, (b). what carrying anopheles are present and in what parts of the area. Next start dissecting sample catches of known anopheline carriers. Record oocyst rates=percentage shewing oocysts in gut : sporozoite rate far more important,=percentage shewing malarial sporozoites in salivary glands.

We can now report somewhat as follows :—

Malarial probably of old standing in Dustypore. Spleen, endemic and gametocyte indices as shewn. Spot map shews (a). distribution of human carriers and reservoir of infection, (b). anopheline carriers of the area, when and where breeding. Oocyst and sporozoite rates for the different species found to be so and so. Reasons for sudden epidemic prevalence of malaria in Dustypore :—(a). Large reservoir of malaria carriers in young children of locality. (b). Arrival in area recently of non-immunes in large numbers, e.g. perhaps imported coolies working on railway line. (c). Infection is being carried, e.g. by A. culicifacies and A. stephensi, whose chief breeding spots are shewn in map. Seasonal incidence of them is as shewn. Recommend.—(a). All human carriers be thoroughly treated with cinchona febrifuge, especially during next cold weather off-season. (b). At times of year as shewn, when wet bulb temperatures are suitable for malarial transmission, intensive measures be carried out against A. culicifacies and A. stephensi; and their breeding places, as shewn, be intensively dealt with. (c). General educational campaign with lantern lectures etc. in the locality to teach people why malaria now so prevalent locally, and how to deal with it. (d). Usual pious hope that generally filthy condition of Dustypore be improved by introduction of better sanitation.

This a much better plan than to take a lot of kerosine oil and more eyewash and work wildly and indiscriminately against all and sundry mosquitoes, mostly probably Culex, in the area. Funds being limited, a properly directed campaign based on a thorough knowledge of local factors will do a hundred times more than vague, random measures all the year round, too widely scattered to achieve useful results. May now leave Dustypore and go next door to Dirtierbad and find epidemic malaria from totally different factors : non-immunes may be local residents, disease may have been recently imported from outside, carrying Anopheline species may be different, seasonal incidence different. Dont believe there is any royal road to exterminate Bengal malaria—such as universal, controlled flooding, as recommended by Bentley :—believe each area must be enquired into on its own merits.

Blackwater Fever.

In India especially in the Dooars, Assam, Bengal. Once common in the Panama Canal zone, has now disappeared from it, with disappearance of malaria. Actual condition is terrific destruction of R. B. Cs., haemoglobinaemia, with consequent haemoglobinuria, urobilinuria and albuminuria. Even healthy liver unable to deal with excessive Hb. destruction products. General post mortem findings in the disease are those found in M. T. malaria. But blackwater fever has also occasionally been found associated with B. T. and quartan parasites. Occurs only in those who have resided a long time in tropics. Jaundice sets in early and is intense with very marked parallel destruction of R. B. Cs. Some cases lose two million R. B. Cs. per c.mm. in two days. Serum tinged. Hb. reduction parallel to R. B. C. reduction. Spleen enlarged and tender. Urine shews spectroscopically absorption bands of methæmoglobin, or more rarely of oxyhæmoglobin. Marked prostration with clear and anxious consciousness. Blood coagulability, alkalinity and pH reduced. Schlesinger's test for urobilinogen in urine positive. Occasionally hæmatoidin crystals in urinary deposit.

Dudgeon, 1920, has demonstrated presence of active hæmolysins in tissues and urine of blackwater fever cases, which he was unable to find in malaria cases or other diseases. No evidence that R. B. Cs. shew extra fragility in the disease. No evidence of existence of auto-haemolysins. Bile pigment occurs in plasma of fatal cases. Post mortem necrosis of Malpighian bodies in spleen very striking.

Theories as to causation : at present nothing proved, " pays your money and takes your choice." (a). It may be due to some, as yet undiscovered, specific parasite. Symptoms exactly like those of piroplasmosis in animals. Sambon considers it=human piroplasmosis. Several workers have described piroplasma-like organisms in blood, but nothing proved. (b). May be merely hyper-virulent M. T. malaria. Stephens takes this view. Malaria parasites found in 95% of cases whose blood is examined the day before hæmoglobinuria sets in, and in 62% of cases whose blood examined on the day the blackwater occurs. Only occurs in areas where malaria is rife. This a weak argument : it used once to be applied to explain that kala azar was probably a form of chronic malaria. Experience in Macedonia during war shews that transient hæmoglobinuria is not uncommon in severe M. T. cases, but such cases were not like true blackwater fever. (c). That it is quinine hæmoglobinuria, occurring in persons especially susceptible to quinine poisoning. Quinine normally circulates in blood as base, no matter in what form administered. But if alkali reserve be lowered, quinine may circulate as acid salt in blood, causing hœmolysis of R. B. Cs. (d). The usually accepted view is that it (b) plus (c). Usual history of blackwater fever in Europeans at least is (a) occurs only in malarial saturate, who (b) is accustomed to periodic attacks of malaria, and who (c) takes a dose of quinine, and then (d) black water follows. Have seen two instances of this, but have also seen it develop in a Khasi prisoner in Shillong jail, where no parasites found and only quinine taken was weekly prophylactic dose.

Great necessity for careful study of blackwater fever cases, to elucidate true etiology of the disease and settle controversy.

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LECTURE 14.

PLASMODIA, CONTINUED : PROTEOSOMA, HAEMOPROTEUS, LEUCOCYTOZOA.

Plasmodia strictly specific to their own hosts. In addition to malarial parasites of man, in monkeys P. kochi of chimpanzee, P. pitheci of ourang-outang, P. inui of Macacus tribe, P. semnopitheci of hunuman, the small ape of Assam. Cycles exactly like those of malarial parasites, and carried by anopheles mosquitoes. In dogs Plasmodium very much resembling P. vivax, and with Schüffner's dots described by Castellani. P. vassali of Indian ground squirrel, Patton. Plasmodium of Indian buffaloes. Four species of bat plasmodia described, but doubtful whether true parasites or merely R. B. C. changes.

Proteosoma, sub genus. Plasmodia of small birds. Life cycles exactly like those of malarial parasites : transmitted by Culex mosquitoes. Munnias, Java sparrows, goldfinches, thrushes etc., infected. Young trophozoite ring near pole of nucleated R. B. C. As it grows it pushes the nucleus of the R. B. C. askew, this very characteristic of Proteosoma infections. Schizogony rosette shews 8 or more merozoites. Produces much black haemozoin pigment. Not infrequently double infection of cell, rosette at each pole. Internal viscera very heavily pigmented in Proteosoma infections. Gametocytes spherical or oval forms at poles of R. B. Cs. When maturing throw out tiny bit of polar chromatin. Male has pale blue cytoplasm with big, diffuse chromatin mass : female cytoplasm stains deeply and with small but deeply staining chromatin mass. Sexual sporogony cycle in Culex just like that of malaria in anophelines. Oocyst very deeply pigmented. Discovery of Proteosoma cycle of in Culex by Ross led to discovery of Anopheles cycle of malarial parasites. Travelling vermicules in Culex mid gut at 12 to 15 hours after feed : oocysts from 2nd day onwards, sporozoites collecting in salivary glands from 5th day onwards. Some workers consider infection also transmitted from mosquito to its progeny, but this probably not so. The Sergent brothers have recently shewn that blood of birds who have recovered and where parasites cannot be found still infective on intravenous injection into fresh birds. Christophers has shewn that bird already infected with Proteosoma will take fresh superinfection if bitten by infected mosquitoes. Grassi in Italy described "black spores" as infecting mid gut of Proteosoma-infected Culex, and even in Proteosoma oocysts, but shewn by Christophers that this was a Nosema, (vide later). Proteosoma and Haemoproteus very favourite Haemosporidia for laboratory study: many malarial problems can be studied by analogies in Proteosoma and Haemoproteus.

Hæmoproteus, vel Halteridium genus. Live in nucleated R. B. Cs. of passerine birds, such as pigeons, owls, paddy birds, parrots, Java sparrows, munnias, etc. Simultaneous infection in a bird with Proteosoma plus Haemoproteus not at all uncommon, and failure to realise this fact has led many workers on the species astray. Accounts of life cycle of Haemoproteus now current in the text books entirely wrong. Aragao, 1908, described life cycle as follows :—1. parasite, he says, at first lives in large mononuclear leucocyte : then invades endothelial cell in lung : where it undergoes very complicated schizogony cycle with production of millions of merozoites. 2. Some of these enter R. B. Cs. and pass to peripheral blood and become gametocytes. 3. Infection transmitted from pigeon to pigeon by bitting Hippoboscid fly, Lynchia maura. Supposed that fly feeds on pigeon, ingests gametocytes, these conjugate in its mid gut, zygote results. Then comes extraordinary statement: fly next supposed to infect a clean pigeon by injecting the zygote into it. This life cycle almost incredible on the face of it. Most unlikely that different forms of the parasite would inhabit in turn leucocyte, endothelial cell, and R. B. C. Further, function in life of a Haemosporidial zygote is not to be injected into the vertebrate host, but to become transformed into an oocyst in the insect host, and infective forms almost certain to be sporozoites.

True life cycles of Haemoproteus columbae given by Acton and Knowles, 1914, for schizogony cycle and by Mrs. Adie, 1915, for sporogony cycle.

Literature on Haemoproteus almost incredibly muddled. Woodcock, 1911, described union of cytoplasms of gametocytes when lying together in same R. B. C. Most unlikely. Other workers have described male and female gametocytes

as conjugating whilst within the same R. B. C., lying side by side. This utterly impossible : gametocytes dont conjugate : it is gametes which conjugate, and—further—the Haemosporidia do not conjugate in the vertebrate but in the invertebrate host. Schaudinn's classical paper, 1904, made one stupendous muddle of the whole affinities of the Haemosporidia. Schaudinn worked with the little owl, Athene noctua. This bird has no less than six blood inhabiting parasites. : viz. :—1. a haemoproteus. 2. a small trypansoome. 3. a bigger trypanosome ? identical with the small one, or different ?. 4. a leucocytozoon. 5. a proteosoma, and 6. a spirochaete. Result when biting flies fed on such a Chinese puzzle of a bird almost incredible confusion. Schaudinn described conjugation of haemoproteus gametes, which then become transformed into trypanosomes, which were male, female or neuter. Injected by fly into fresh owl: whereupon on injection the trypanosomes changed back into halteridia. Also supposed that Haemoproteus, Trypanosoma, and spirochaetes were all developmental forms related to one another. The student will find the whole of this dreadful muddle faithfully repeated today in most text books, e.g. Castellani and Chalmers give it in detail.

Results of Schaudinn's paper disastrous influence on protozoology for nearly twenty years : even today is leading investigators astray. E.g. workers raising such absurd problems, as :—Are spirochaetes in reality derived from trypansomes or from leucocytozoa or both ? Is the parasite of syphilis really a "leucocytozoon" whose resting phase is in the large mononuclear leucocytes ? Have trypanosomes sex ? Are the Haemosporidia the same as the Haemoflagellates, or diferrent ? Having made confusion confounded in the entambae, Schaudinn's second great paper, the one quoted above, made confusion worse confounded with regard to the whole of the blood inhabiting protozoa. To quote Minchin he created an unnecessary and 'veritable riddle of the Sphinx.'

Work of several distinguished protozoologists, especially Minchin and Woodcock, and the Sergent brothers finally cleared up the muddle. Full discussion will be found in Chapter 15 of Minchin's "Introduction to the Study of the Protozoa."

True schizogony cycle of H. columbae, Acton and Knowles, 1914, is as follows :—Youngest form met with is tiny ring trophozoite, just like a proteosoma ring, only placed laterally near one end of the R. B. C. nucleus, in cytoplasm of R. B. C. As it grows the R. B. C. becomes pale and distended. It is caught in a capillary blood vessel in the lung as it passes through. Here the R. B. C. goes to pieces, leaving the growing trophozoite stranded in and blocking the lung capillary. Next schizogony sets in the chromatin divides into 2, 4, 8, 16, 32 etc. bits, until finally we have a sort of pseudo-cyst= the blocked and distended and distorted capillary, filled with myriads of little merozoites, each invested with a thin membrane. This now ruptures into the blood stream, the circulation through the capillary being re-established. Some reenter R. B. Cs. to repeate the schizogony cycle others enter R. B. Cs. to become converted into gametocytes and get into the peripheral circulation. The asexual forms are never pigmented, the sexual forms are deeply pigmented with haemozoin.

Kasauli pigeons repeatedly examined, but failure to find the cycle. Cycle finally discovered only in heavily infected birds brought up in the spring from the plains, from Umballa to Kasauli. Usually the infection causes the bird no trouble, but very heavily infected birds may die with broncho-pneumonia. Sections of such heavily infected birds' lungs stained with iron-haematoxylin are very pretty objects : shew lung tissues riddled with the pseudo-cysts of different shapes and sizes, according to character of blocked capillaris. It was these pseudocysts which Aragao mistook for infected endothelial cells.

Youngest gametocyte forms are also young rings, but haemozoin pigment forms early. As they grow they curve around the lateral aspect of the R. B. C. nucleus, whence name "Halteridium" from halter-like shape. In many ways recall the crescents of P. falciparum. Adult male gametocyte has pale blue cytoplasm, very clear and faintly staining, chromatin as loosely wound, badly staining mass in centre : pigment as 3 to 7 intensely brown black blobs at each pole. Female halteridium gametocyte is thinner and with more irregular edges and more pointed : cytoplasm stains a deep blue : chromatin present as a tightly wound, deeply staining mass in centre of parasite, pigment as fine brown-black grains scattered throughout the parasite. By degrees the schizogony cycle in the lung dies down, leaving only gametocytes. Asexual forms rarely encountered in the peripheral circulation, which as a rule, only shews gametocytes of all ages. By degrees peripheral blood swarms with gametocytes.

Sporogony cycle, Mrs. Adie, 1915. Transmitting fly, Lynchia maura, lives in pigeon's nest. Ingests blood. In its mid gut the gametes conjugate to produce a zygote= ookinete=travelling vermicule. Whole of the cycle is IDENTI-

cal with that of malarial parasites in Anopheline mosquitoes. Most unfortunately in publishing, Mrs. Adie did not publish plates, as cycle so exactly similar to that of malaria, but her preparations studied at every stage by Acton and Knowles, and zygotes, oocysts, pansporoblasts, entry of sporozoites—which closely recall those of malaria—into salivary glands and their passage down the salivary ducts in fresh dissections seen. Mrs. Adie's success in proving this cycle entirely dependent upon her unique skill as an insect dissector. Probably previous workers never saw the true salivary glands of the fly at all. The salivary glands lie in the ABDOMEN of the fly, close to the mid gut. From them two fine ducts pass forwards, crossing in waist of the fly, and enter thorax. Here each is next distended into a pear-shaped salivary reservoir with thick muscular walls. This organ functions as a salivary reservior and pump, and was probably mistaken by earlier workers for the true salivary glands. Had they mastered the true anatomy of the fly they would have found the cycle.

This work all published in 1914-15, yet even now ignored by the text books, which till go on reproducing Aragao's statements and even Schaudinn's muddle.

One additional note. In Haemoproteus orizovorae Anschutz, 1910, described schizogony as taking place in the infected R. B. Cs. in the peripheral blood. His original plates not very convincing : suggest rather bad fixation. Somewhat similar appearances seen in halteridium of the buzzard by Mrs. Adie in 1920, but interpretation doubtful. Am most anxious to get hold of paddy birds to try and get at the true facts with reference to this species, but unfortunately paddy bird so valued in Bengal for its fat that almost impossible to purchase them in Calcutta.

Life cycles being as above, question now arises whether the genus Haemoproteous ought not now to be merged in genus Plasmodium, as subgenus similar to Proteosoma. When both Proteosoma and Haemoproteus infections simultaneously present in the same bird, can differentiate them by the fact that Proteosoma grows at pole of R. B. C. and pushes its nucleus askew, whereas Haemoproteus grows laterally beside the R. B. C. nucleus and is halter shaped.

Haemoproteus infections very common and even water fowl of Calcutta Zoo found infected. Sambon has described H. mansoni of the grouse, with transmission by grouse fly : but—misled by Aragao's work—he considers that fly injects zygote into the grouse.

Genus Hæmocystidium, Castellani and Willey, 1904. Are big Proteosoma-like parasites inhabiting nucleated R. B. Cs. of snakes, tortoises, and reptiles. Greatly distend and distort infected R. B. Cs., and push nucleus askew as does Proteosoma. Schizont rosette shews four big merozoites. Life cycles like those for Proteosoma, except that infection transmitted by leeches. Paper by Shortt, Indian Science Congress, 1922 (not as yet published as far as known), bringing forward evidence that genus Haemocystidium should be included in and regarded as sub-genus of Plasmodium.

True Lucocytozoa. Genus Leucocytozoon. Must be carefully distinguished from the false leucocytozoa=haemogregarines of warm blooded mammals, inhabiting mononuclear leucocytes. True Leucocytozoa are parasites of birds, especially of partridges, pheasants, owls, grouse, hawks, turkeys, guinea fowl, etc. Found in swans also in Calcutta Zoo. Chief work on the genus that by Fantham, 1910, Wenyon, 1910 Sergent brothers, 1907, Sambon and Seligman, 1907. Specially recommend for good account Wenyon's 1910 paper.

True nature of host cell not certain, see later. The schizogony cycle, according to Sambon, occurs in the spleen, young trophozoite inhabiting certain mononucleate cells in the spleen capillaries. Oval, 8 by 12 μ, and with single nucleus. It grows up to form a schizont rosette, with from 12 to 20 merozoites. The infected cell now ruptures : merozoites set free : either re-enter cells locally to repeat schizogony or enter certain mononucleate cells to pass into peripheral blood.

The parasite possesses the peculiar property of very greatly distorting the host cell which it inhabits. The infected cell in the peripheral blood becomes drawn out into a characteristic spindle form with both ends terminating in a sharply pointed tail. For this reason impossible to tell true character of greatly distorted host cell, ? pale and decolourised R. B. C., ? large mononuclear leucocyte ? endothelial cell free in plasma. Probably the first.

Only gametocyte forms met with in peripheral blood. Three types : (a) young immature gametocytes, oval, with central nucleus lying close beside the host cell nucleus. As it grows it squashes the host cell nucleus against the host cell envelope, at first squeezing it into a dumb bell shape, then into a mere ribbon of chromatin, scarcely visible. (b). Macrogametocytes, 14 to 20 μ long, by 10 to 16 μ broad, with deeply staining cytoplasm, full of reserve food particles, and with deeply staining nucleus, inside which is a very striking and large and deeply staining ex-centric karyosome. (c) Microgametocytes

18 to 17 μ long by 6 to 12 μ broad, with pale staining cytoplasm, and large, granular and badly staining big nucleus. In addition to these we get occasionally in films from the viscera and heart blood similar forms which, however, do not distort the host cell ; spherical forms.

Transmission of leucocytozoon of the red grouse by the grouse fly, Ornithomyia lagopodia, Sambon. When fly ingests gametocyte-containing blood, gametocytes leave cells. Male ex-flagellates in manner similar to malarial crescent, female leaves host cell also and becomes spherical, motionless, rounded form. Fertilisation just as for malarial parasites, with formation of a zygote which is curious, long, twisted, wormlike, vermicule form with writhing, gliding movements. Sambon, misled by Aragao's work on Haemoproteus, states that fly next probably injects zygote into fresh grouse, but this most unlikely. Probably rest of sporogony cycle like that of malaria with sporozoite-formation : remains for someone to investigate.

The leucocytozoa do not form pigment. Name " Leucocytozoon" one of the most abused in protozoology. Eg. H. Ross describes the Kurloff body of the guinea pig, see later, as a leucocytozoon, he means haemogregarine, but it actually is neither. Other curious views as to parasite of syphilis being really a leucocytozoon, also wrong. Schaudinn's 1904 paper has much to answer for.

Excellent review of the Haemosporidia in chapter 15 of Minchin. The different genera of the Haemosporidia shew close affinities, whilst on the other side they shew resemblances to the intestinal gregarines and coccidia, modified by insect transmisison instead of transmission by contaminative methods. Fundamental features are that they all have schizogony multiplication cycle in the vertebrate host : in most, e.g. Haemogregarines, Haemoproteus, P. falciparum, and Leucocytozoon in the internal viscera : in some, e.g. P. vivax, P. malariae, Proteosoma, Haemocystidium in peripheral blood also. Some produce haemozoin pigment, e.g. malarial parasites, Proteosoma, Haemocystidium, others have none, e.g. Haemogregarines, Leucocytozoa. All are true Telosporidia, with spore formation being the end phase of the life cycle : in all as host becomes resistant the asexual schizogony cycle dies down, to be replaced by gametocyte formation in preparation for passage through a biting insect to the next vertebrate host. In all in response to the necessity of getting into the biting fly, the gametocytes make especially for the peripheral blood stream. Note the supreme necessity in examining protozoal blood infctions of studying the internal viscera as well as the peripheral blood. Their pathogenicity varies from the harmless haemogregarines of lizards and toads, to the deadly piroplasmata of animals, but in general the tendency is towards mutual accommodation of host and parasite, as is the rule with most parasitic protozoa.

Practical Notes. Sectioning and Staining of Tissues for Protozoa.

1. The tissue must be obtained AS FRESH AS POSSIBLE. The protozoa rapidly disintegrate after death of the host, and a few hours after death the material is almost useless. If much blood rinse rapidly in saline. Drop slices not more than ¼ inch thick into at least ten times as much Zenker's fluid. Place for 24 hours in the warm incubator.

2. Wash thoroughly in tap water.

3. Soak for 24 hours in 70% alchool. Tissues can be left in this almost indefinitely until time permits of embedding and cutting.

4. For embedding the best procedures are the acetone method and the use of Hearson's vacuum embedder. Transfer the tissue from 70% alcohol to a pot of acetone kept in the warm incubator. Leave for half an hour. Transfer to fresh acetone ; leave for half an hour. Transfer to a third pot of acetone : leave for half an hour.

5. Transfer to absolute xylol : keep in warm incubator for half an hour.

6. Place in melted paraffin,—melting poing 54°C for all work in tropical climates, except hill stations,—in the vacuum embedder. Exhaust air and leave for half an hour to an hour. The chief point of the vacuum embedder is that all air is extracted from the tissues and thorough permeation of the paraffin assured.

7. Embed in square block of paraffin in the usual way. For section cutting either the Spencer Lens rotary, Minot or new model Cambridge rocking microtome is best. Cut the sections at 2 to 4 μ.

8. Float the ribbon of praffin sections on warm water. Pass a clean slide under the ribbon of sections and withdraw so that the sections adhere to the slide. Place the slide vertically in the warm incubator for 24 hours, when the sections will adhere to the slide. The use of albumin fixative is NOT recommended,

9. Xylol to remove paraffin.

10. Absolute alcohol, followed by rectified spirit.

11. Transfer to 70% alcohol tinged a deep mahogany brown with iodine crystals. Leave till the sections are a deep brown : the mercury in the fixative is changed to mercuric iodide.

12. Soak in rectified spirit till all the brown colour is gone =the mercuric iodide is dissoved out of the tissues. Essential that this should be done completely.

13. Pass down the descending alchools, 70%, 50%, 30% to distilled water, and soak well in distilled water. The sections are now ready for staining. The two best methods are :—

14. A. Haidenheim's Iron Haematoxylin Method.

1. Soak the sections in 4% solution of iron alum, (violet and not green crystals), for about four hours to mordant.

2. Rinse rapidly in distilled water.

3. Flood the slide with Shortt's carbolised haematoxylin stain, and repeat two or three times. Continue till the sections are a jet black.

4. Wash in water.

5. Decolourise in one per cent. iron alum solution, watching the process under the microscope. At the moment when the black stain has disappeared from the cytoplasmic structures, but is still in the nuclear structures, remove.

6. Wash thoroughly to remove every trace of iron alum in running tap water.

7. To counter-stain stain for a minute or less in either one per cent. watery eosin, or Bordeau R. 1. gm., absolute alcohol 10 c.c., water 90 c.c. sloution.

8. Wash thoroughly in water.

14. B. Wolbach's modified Giemsa stain for tissues.

(see Wolbach, S. B., 1919. Journal Med. Research. XLI. No. 1. pp. 75 to 78.)

The distilled water used must be acid-free, and from two to four drops of a 0.5% solution of sod. bicarb. in distilled water should be added per 100 c.c. The stain used is therefore :—Mix undiluted Giemsa's stain 2.5 c.c. : Merck's pure methyl alcohol 8 c.c. : 0.5% sod. bicarb. Solution 2 to 4 drops : distilled water 100 c.c.

1. Pour the stain over the sections : leave half an hour.

2. Repeat 1.

3. Repeat 1. Leave the sections in this now for 12 to 18 hours. The sections should now be heavily over-stained.

After staining by either method :—

15. Wash the stained sections thoroughly in distilled water.

16. Pass in succession through 30%, 50%, 70%, rectified and absolute alcohol. With the Giemsa stained sections this must be done very rapidly before the stain has time to be taken out of the sections. Wolbach recommends going direct into and differentiating in 95% ethyl alcohol..

17. Soak in absolute alcohol for iron haematoxylin sections, or after rapid dehydration in it for Giemsa stained section, transfer to a mixture of equal parts absolute alcohol and xylol. Soak.

18. Transfer to absolute xylol, and soak till clear.

19. For iron haematoxylin sections mount in balsaam, for Giemsa stained sections in cedar wood oil, ringing the slide with gold size.

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LECTURE 15.

THE NEOSPORIDIA. CNIDOSPORIDIA, MYXOSPORIDIA, MICROSPORIDIA, NOSEMA. SARCOSPORIDIA. SARCOCYSTIS. HAPLOSPORIDIA, RHINOSPORIDIUM KINEALYI.

Neosporidia are second big sub-division of Sporozoa. Characterised by commencing to form spores whilst vegetative life is still going on, so that vegetative and sporogony cycles overlap. The vegetative forms are simple, spores complex, and the group probably derived from some amoeboid ancestor.

They commence life as little amoebulae, planonts or wanderers. In some species this is succeeded by a plasmodium phase. (Here note this use of term plasmodium, not as name of genus, but for sheet of individuals with fused cytoplasms). This fusion is merely cytoplasmic and temporary—plasmogamy—and merely represents among the protozoa a further advance on colony formation. The nuclei do not fuse and the plasmodium is studded with individual nuclei, each representing nucleus of one individual. Such plasmodia shew clear marginal ectoplasm, granular endoplasm. Can split up again into individuals or into smaller sheets of several individuals apiece, and such individuals or smaller sheets fuse again into bigger ones.

Neosporidia divided into :—(a). *Cnidosporidia*, which possess polar capsules, and (b). *Haplospori dia*, which are vaguely defined group with no polar capsules. Polar capsule one of the most striking structures met with in protozoa. Hollow, pear shaped with dense, thick chitinous wall, and tiny pore at anterior end. Inside lies coiled up, pre-formed explosive filament, like coiled up mainspring of a watch. Filament of very great length. When spore ingested into gut of new host, polar capsule explodes, polar filament is shot out with great force, and by tiny bulbous tip adheres to wall of gut. Anchors spore to gut wall, and from within spore the tiny amoebula now creeps out, makes for gut wall, gets to it and penetrates into it : new host thus infected. Four nuclei concerned in the polar capsule: two go to form the tough chitin wall, one to form the explosive filament, fourth left as spore nucleus of amoebula within spore.

Cnidosporidia divided into :—

1. ***Myxosporidia.*** Order with immense number of genera. Tissue and gut parasites of fish, amphibians, reptiles. Also in gall bladder and urinary bladder of fish. Even infect the C. N. S., as with Myxosporidia of salmon and trout. May be seen in tissues as just visible pearly white spots=plasmodia, or the infection may so riddle the tissues that cannot say what is plasmodium and what tissue. Or may be big plasmodia with ectoplasm and endoplasm, and pseudopodial movement.

After ingestion of spore it explodes in gut. Amoebula emerges and makes for gut wall of host. Gets through and into blood and lymph stream and penetrates into every tissue. Divides by binary fission repeatedly so that immense broods are formed. Individuals in tissues may fuse by plasmogamy to form plasmodial sheets, and such sheets repeatedly breaking up and re-fusing. Plasmodia studded with multiple nuclei; also in endoplasm crystals, fat globules, pigment granules etc. No food particles, feed by osmosis from tissues. This constitutes the vegetative phase, and tissues get simply riddled with infection.

As nourishment used up and whilst multiplication by schizogony still occurring, sprogony sets in. Spores formed within the plasmodium. Thus Myxidium liebkuhni of the pike's bladder, in summer active schizogony with bladder carpeted with slimy plasmodia of orange colour, in winter sporogony sets in in these plasmodia. Myxobolus pfefferi of the pike as example. One of the many nuclei in the plasmodium plus surrounding cytoplasm gets shut up in a clear vacuole within the substance of the plasmodium,=cell shut off from, but contained within plasmodium. It divides into two cells, one with a big, the other with a small nucleus. Cell wall between two now breaks down but nuclei do not fuse, cytoplasm only fuses. This peculiar structure called a gamont. Note stages so far: planont, plasmodium, gamont.

The two big nuclei now each divide into 4, 8, 12 big nuclei =in all 14 nuclei in cell, 12 big, 2 small. Two small nuclei pass to periphery and go to form chitinous wall of spore. Of the 12 big nuclei 8 go to periphery and 4 remain central. Four central nuclei=gametes. Next contents of cell, cell=pansporoblast, divide into two equal halves. We have now (a) outside dense chitinous envelope of the pansporoblast: (b) inside two sporoblasts, each with 6 nuclei, 4 peripheral, 2 central. At this stage the tough walled spore is passed out in the faeces, or, often, the host having died, its tissues are eaten by fresh host. Or fish host may die and its tissues disintegrate in water, setting free degenerating plasmodia studded with myriads of complicated spores, and these be ingested by fresh hosts.

Infection acquired by new host either by contamination of water or by eating former infected host. When spore gets into gut of new host polar capsule explodes and anchors spore to gut wall : the two central nuclei fuse, and finally a uninucleate amoebula crees out to make for gut wall and infect new host. No intermediate host necessary : infection via contaminative cycle. To summarise cycle : amoebula, planont ; plasmodium with schizogony, plasmogamy, plastogamy, plastotomy : sporogony within plasmodium : spores set free : ingested : infection of new host. Terrific epidemics among fish often caused by Myxosporidia.

2. *Actinomyxidia.* Parasites of oligochaete worms of both fresh and salt water. No plasmodium phase : remain as single amoebulae, dividing by binary fission. Spores are trimetrically symmetrical : each has 3 polar capsules, and contains not one but innumerable amoebulae or sporozoites.

3. *Microsporidia* are parasites which are of immense economic importance. Causes of tremendous loss to agriculturalists. Nosema bombycis the cause of pébrine disease of silkworms : Nosema apis the cause of bee disease. Classical work of Pasteur, 1865, on N. bombycis : discovered life cycle, methods for detection and prevention of infection, and within a few years French silkworm industry had increased annual income by 50 million frances a year and the silk industry of the world saved from ruin. Final historical experiment at the Villa Vincentina. Recent work of C. M. Hutchinson, 1917-20, at Pusa on pébrine in India : if his recommendations be adopted, Indian silk industry, which is now almost moribund, may yield crores a year to the country.

Life history of N. bombycis. Silkworms infected by eating mulberry leaves contaminated by faeces of infected worms full of spores. In mid gut, posterior portion, the polar capsules of the spores explode, anchoring spore to gut wall. Amoebula creeps out, wanders to gut wall, becomes planont, and penetrates through gut wall. No plasmodium phase, but incessant multiplication by binary fission. By degrees the entire silkworm becomes riddled with the disease, every tissue infected except the cuticle, even the nerve ganglia riddled ;

get worms which are literally bags of disease and yet seem healthy and feed vigorously. If however larvae heavily infected in early life growth stunted. Infection persists as silkworm passes into cocoon stage and in this stage multiplication very active indeed. Passes into mature moth and from mature female moth into its ova, planonts wandering into and infecting the ova in situ. Hereditary transmission. Pasteur shewed : if you want to know whether moth is infected and its seed will be infected or not, nothing easier. Select sample cocoons of the brood. Place in warm incubator when young, immature moths will be born. Take these, crush in pestle and mortar, examine under x 600 magnification for the characteristic black spores, : (whence name of disease, pébrine, from black pepper-grain like markings of infected worms and moths.) If moths infected we know that ova are infected : cannot be kept for next year's silkworms. If test moths healthy, seed presumably healthy. Pasteur's work a striking example of early day research in protozoology with economic results of immeasurable value to mankind. French government now sell two types of guaranteed seed : (a) in which every moth has been examined and no pébrine detected, and (b) in which a proportion of moths have been examined and no pébrine detected.

Sporogony cycle. Growing trophozoite develops direct into spore. Vacuole forms in trophozoite and two bits of chromatin budded off from nucleus and go to form the tough capsule. Whole interior of spore occupied by one enormous polar capsule to which third bit of chromatin goes to form polar filament. Finally within the spore is the amoebula or sporozoite, present as a thin band of cytoplasm containing two nuclei wrapped equatorially around the huge polar capsule. Such spores passed in myriads in faeces of infected worms : gross contamination of mulberry trees : whence enormous epidemics. Hereditary plus contaminative infection cycles. In India as Hutchinson shews matters much worse. Not one but four or five silkworm generations a year. He also shews that Pasteur's method of crushing moth too crude for India : and tends to be replaced by even cruder and more contaminative method of crushing test moth in bit of paper which is afterwards thrown on to floor to carry infection still further. The chief focus of infection in the worm and moth is at the posterior part of the mid gut, and if this be dissected out and examined, percentage of positive findings greatly increased and method much more accurate than Pasteur's. If only India would adopt his proposals Indian industry might have immeasurable future before it. But "what can do?". Government instructed Pusa

Institute to supply guaranteed pébrine-free seed to all enquirers : one received such a consignment ; replied by post " The worms received in excellent condition, in fact some had laid eggs during transit in post." Hutchinson's work well worth study by protozoologists : technique superb and microphotographs unrivalled.

In Nosema apis, the parasite of bee disease, infection confined to gut, but sporogony cycle similar, and infection via contaminative channel. In general the Microsporidia are parasites of insects and arthropods and fish. Crayfish, flies, Ephemerid larvae infected, and one species even parasitic upon a Gregarine itself parasitic, splendid instance of hyperparasitism. Strickland, 1922, discovered Nosema infection of Anopheles rossi larvae in Calcutta near the School.

4. *Sarcosporidia* live in flesh and muscles, whence their name. Are especially parasites of striped muscle in pigs, sheep, horses, cattle, buffaloes, monkeys, seals, birds-rarely-, rabbits, rats. Sarcocystis muris the cause of terrific epidemics among rats. Very rarely they occur among reptiles.

In man Sarcocystis infection described by Lindemann, 1878, in myocardium : 1887 by Koch : Kartulis, Sudan cases ; Rosenber case in 1902 : Baraban, 1894, case of infection of laryngeal muscles : Darling a case in the Barbadoes. Quite likely that infection in man is much commoner than usually supposed.

Sole genus is Sarcocystis. The place to see it is in Indian butchers' shops. Infects muscles, especially those of skeleton, tongue, larynx, and diaphragm. Seems to cause no inconvenience to host unless essential muscle, such as myocardium, involved. In infected meat in butchers shops we see white, opaque, cylindrical tubules, 16 or more mm. in diameter= Meischer's tubes=pansporoblasts. (Terminology here vague as first described by veterinary surgeons with little knowledge of protozoology.)

Infection acquired via gut. Planonts wander through gut wall into lymph stream and are disseminated to striated muscles throughout body. Grow in longitudinal striated muscle fibres, but cycle of S. tenellae bubali of the buffalo still imperfectly known. In the infected muscle fibre we finally get the ripe pansporoblast=Meischer's tube, crammed with sporozoites. These sporozoites (? merozoites) are crescent shaped or sickle shaped, 1 to 21 μ long, with single nucleus and granular cytoplasm. They have a peculiar gliding, gregarine-like movement. Are termed Rainey's corpuscles, and on rupture of the

Meischer's tubes are scattered, probably via the lymph stream, through the body as well as locally to infect fresh striated muscle fibres.

The fullest life history known is that of Sarcocystis muris of the rat. Here infection occurs via the gut and contaminative cycle. The planonts wander into the muscles from the gut and settle in the striped longitudinal muscle fibres. They grow, retaining the shape of the enclosing muscle fibre, into long cylindrical sacs, each containing a plasmodium, 25 μ or so long, studded with nuclei. Each nucleus next surrounds itself with cytoplasm and becomes a sporoblast. The whole cylindrical tube=pansporoblast, 450 μ long, and its cavity is cut up by transverse and longitudinal partitions into chambers, more or less incomplete. Later all partitions break down, and inside each sporoblast the nucleus divides, and two sickle shaped sporozoites=Rainey's corpuscles in sarcocystis infection of cattle, are formed, 3 to 14 μ long. Each sporozoite is enclosed in delicate investing membrane : has a gliding gregarine-like movement : single prominent central nucleus, and at anterior pole traces of spiral structure=rudimentary polar capsule.

When pansporoblast ruptures, sporoblasts also rupture, setting free sporozoites. These are disseminated by lymph stream. In cattle infections they have also been found by Castellani in blood stream.

Man infected by eating half-cooked diseased beef and meat. In S. muris mice infected experimentally by feeding them on dead bodies of infected mice, pectoral muscles found infected on 50th day afer feed, Meischer's tubes present. Cattle, however, are not carnivora and could not be infected via this route in nature. Puzzle as to how the infection is spread among cattle. As sporozoites after rupture of Meischer's tubes present in circulating blood, possibly infection transmitted among cattle by some biting cattle fly : possibly also via faeces and contaminative cycle ? In infected mice the faeces remain infective for over a month after being passed.

Haplosporidia.

Very vague group and include a whole jumble of Sporozoa with little known life histories. Divided into two sub-groups, (a) Oligosporuleae with a plasmodium which divides direct into sporoblasts, each producing one spore. Here chief genus is Ichthyosporidium which causes fatal epidemics in fish, rotifers, worms, etc. (b) Polysporuleae, where plasmodium divides into pansporoblasts, inside which is formed a cluster of sporoblasts. The one human parasite of this group is—

Rhinosporidium kinealyi. Some dispute as to correct name. Seeber, in S. America, 1900, discovered parasite in nasal polypus, but gave incorrect name and failed to place it correctly. In the same year O'Kinealy in Presidency General Hospital, Calcutta had patient with curious nasal polypus studded on surface with white tiny spots. Sent material to Pathology Department, Medical College, Calcutta, where sections shewed cystic structures. Material sent home and studied by Minchin and Fantham, and paper published 1905 : parasite named R. kinealyi. Meantime O'Kinealy read paper in 1903 in London at Laryngoloical Society meeting. Infection now known to affect conjunctivae as well as nose, possibly other mucous membranes. Nair saw several nasal cases, all from Cochin district in 1905. Acton and Knowles got material from nasal polypus shewing infection, about 1913, in course of routine work for Punjab provincial laboratory from the Punjab. Wright, 1922, describes cases in conjunctiva and lacrymal sac and says infection far from uncommon probably. May be met with at any time, if looked for in India, possibly elsewhere.

Polypi concerned are raspberry like in type, and surface shews tiny glistening white spots=cystic pansporoblasts. These are studded throughout polypus tissue but most numerous and largest on its surface. Vegetative phase of parasite unknown but clearly leads to widespread local infection with inflammatory production of granulation tissue and polypus formation. Cysts are end phase of cycle. Outside cyst is connective tissue enveloping capsule. Inside, on cross section are :—1. thin investing envelope proper of pansporoblast. 2. peripheral zone of undeveloped sporoblasts with single nucleus. 3. intermediate zone of sporoblasts undergoing division into sporozoites. 4. central zone of ripe sporoblasts, within each of which is a cluster of 14 oval sporozoites, (? merozoites), each with delicate investing membrane and single piece of chromatin, like a mulberry mass.

When whole structure ruptures, myriads of sporozoites set free on surface of polypus. Some are phagocytosed by leucocytes with formation of small necrotic abscess and discharge of pus from polypus : others recommence life cycle, many probably escape in nasal secretion. How infection is transmitted from man to man not known : ? mechanical transmission in nasal discharges even when desiccated : ? mechanical transmission on pads of flies' feet, flies in India having peculiar predeliction for settling on discharge of nasal and similar discharging ulcers etc. Further work much needed. Please be on look out in India for material : especially for characteristic nasal polypi with tiny white spots on surface.

Affinities of the Sporoza in general. In Neosporidia sporozoite is little amoebula with pseudopodial movements. In Telosporidia, e.g. malarial sporozoite in mosquito's salivary glands, is motile with very characteristic active, swimming, bending tip to tail movement. Ancestor of Neosporidia may have been an amoeboid form, ancestor of Telosporidia a flagellate form. Note how microgametes of some Haemosporidia are flagellate and the existance of flagellate forms of unknown destination in Piroplasma. The two groups almost certainly derived from different ancestries, and only classed together in the Sporozoa for convenience sake.

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LECTURE 16.

THE CILIOPHORA. CILIATA. BALANTIDIUM COLI. BALANTIDIUM MINUTUM. NYCTOTHERUS. COPROZOIC CILIATA.

The *Ciliophora* are last and most highly organised phylum of the Protozoa. They move by ciliary movement. Vast majority of group are free living, but some are epizoic, and a few parasitic, including one or more intestinal parasites of man. Characters :—(a) move by cilia. (b) have two or more nuclei, usually a big, vegetative macronucleus which stains deeply plus a tiny micronucleus which stains badly : (note converse to Haemoflagellates). (c). Unlike other protozoa they multiply by TRANSVERSE binary fission. Are divided into :—(a) the *Ciliata* proper, or true ciliates, which possess cilia throughout their life : and (b) the *Acinetaria* or *Suctoria*, which have cilia only during the earlier part of their life cycle, and are for the most part sedentary forms when adult, with specially developed tentacles for food capture.

Cilia are thin, fine, slender, short hair-like protrusions of ectoplasm, and organs of movement. Much shorter than flagella, and unlike them derived from cytoplasm and not nucleoplasm. Present in the ciliate as a fine, hair-like coating over more or less of the entire body surface. The cilium bends like a bow, with, in addition a slightly spiral twist : and contracts just after the cilium before it and just before the cilium after it, so that optical effect of ciliary movement is like that of successive waves of wind passing over a field of standing corn. (To slow down ciliary movement and study its action add to infusion of ciliate protozoa either a drop of gum arabic solution, or a drop of serum, or of 20% glycerine). Whereas a flagellum pulls its protozoon along after it, cilia row the body of the protozoon about like banks of oars in a Roman trireme.

Cilia have many modifications. Thus :—1. They may combine into stiff tufts, like the bristles of a stumpy shaving brush=cirri. Cirri are like legs, thus Stylonychia usually swims about by means of its cilia, but occasionally turns over on its ventral surface and runs along on its cirri, like a centipede. 2. One row of adjacent cilia, all beating in unison, may fuse into a membranellum. Membranella are usually present in peristome of ciliophoron. They flap about like doors swinging in the wind, and help to waft food particles down the peristome into the cell mouth or cytostome of the ciliophoron. 3. Adjacent rows of cilia, which beat one after another, may fuse into undulating membranes. Here note that undulating membrane of a ciliophoron quite unlike that of a mastigophoron. Latter is periplast of cell raised by contained principal flagellum : former consists of rows of adjacent cilia fused together. Undulating membranes in ciliophora usually present in oesophagus, or in peristome, and serve to drive down food particles into endoplasm of ciliophoron.

The Ciliophora represent the highest degree of evolution of the protozoa : structure of individual very complex, multum in parvo, many of most extraordinary beauty. Yet life cycles relatively simple. Pond water full of free living ciliophora, also sewage effluents especially good material for their study. Classical example, Paramoecium, of which species caudatum almost always found in dung water. Many well known free living genera and species : Nyctotherus with movement like propeller of a steamer, Euplotes and Stylonychia which run about on cirri from time to time, Chilodon with slow steady swimming movement, Pleuronema which puts out from time to time tongue-like membranellum and waves it about to waft food particles into peristome. Amongst the sedentary Acinetaria Vorticella with a contractile stalk common, Campanella like an umbrella turned inside out, Carchesia also with contractile stalk ubiquitous.

Paramoecium as a typical ciliate. Characteristic movements, runs about like a frightened guinea pig. Rapidly rushes forwards, swimming : strikes object : rebounds : rushes off in different direction. Easily cultivated on artifical media. Take chopped straw or hay : make strong infusion in a litre flask : after soaking for a day or two filter into clean flask : bring to boil and boil for ten minutes to destroy all but spore-bearing bacteria. Plug flask with sterile cotton wool and cool. To inoculate examine dung water under dissecting microscope, pick up individual Paramoecia with sterile capillary pipette : wash through two or three changes of cold boiled water to free from bacteria ; inoculate 20 or more into cold hay infusion. They grow in layer under surface pellicle in flask.

Body slipper shaped, covered with vibratile cilia. Thin translucent cell membrane or pellicle. Peristome=funnel shaped depression in surface of body leading down from anterior pole to almost centre of body, where it culminates in small cell mouth or cytostome. In all ciliates the cilia are, in reality, arranged on surface of body like lines of longitude around globe, but in parallel rows with cytostome or cell mouth in their centre. As position of cytostome in different ciliates varies, so also does arrangement of cilia, and therefore characteristic movements of the ciliate. Different species and genera identified in fresh state by their characteristic movements. Paramoecium, Opalina and Balantidium swim straight forwards.

Beneath pellicle comes a layer of basal granules. These give rise to the trichocysts. When Paramoecium wishes to paralyse its prey or to act on defensive it suddenly comes to rest, shoots out its long, fine, hair like trichocysts from entire surface. Trichocysts, unlike polar filaments of polar capsules of the Cnidosporidia, which they otherwise much resemble, are not preformed, but are sudden explosive filaments of ectoplasm. Longer than cilia and appear to arise from a layer of basal granules. Underneath layer of basal granules comes third layer of ectoplasm, = clear line of separation from granular endoplasm. Endoplasm granular and in constant streaming movement round and round inside body of the ciliate. To see these streaming movements add Indian ink or milk to a culture of Paramoecium when the ingested ink or fat particles will be seen to shew the circulatory movements. Endoplasm stuffed with food vacuoles, crystals—often of calcium phosphate,—fat and oil globules,—these very well seen if Paramoecium fed on milk.

Mouth or cytostome is a tiny circular aperture near centre of body. The funnel shaped peristome leads down to it from the pole of the ciliate. As Paramoecium swims forwards food particles are wafted into the peristome, e.g. bacteria, algae etc., then through the cytostome into a short curved oesophagus, in which lies a tiny undulating membrane, and so into the endoplasm of the cell. As food particle passed from oesophagus into endoplasm there forms around it a food vacuole, —like a bubble blown from a pipe,—filled with secretion of digestive function. This food vacuole with contents continues to circulate round and round in endoplasm of the ciliate until digestion is complete. An ingested Bacillus coli is paralysed and rendered motionless in 80 seconds.

Two contractile vacuoles present: they get rid of waste products. One near each pole of cell. As each vacuole swells up it is seen that it is supplied by a system of 6 to 8 little excretory channels. As vacuole swells up it approaches lateral surface, then bursts through pellicle, and returns in empty state to permanent position near pole of cell. Stentor has contractile single vacuole running whole length of cell: Nyctotherus has definite cell anus.

Finally in endoplasm are two nuclei, which do not circulate. Of these the macronucleus is large, stains deeply, and =somatic nucleus. The tiny micronucleus stains BADLY and=germinative and motor nucleus. Through Ciliophora generally this type obtains, but there are modifications. Thus in Opalina innumerable nuclei, each of which may perhaps contain trophic and generative elements : in Vorticella sausage shaped nucleus : in Stentor the macronucleus like a string of beads.

Paramoecium a good example of complexity of structure in a single non-cellular animal. Yet other Ciliophora give even greater extremes of complexity, and some even approach the higher Metazoa in elaboration of function and structure, thus emphasising the equality of the protozoa with the whole super-imposed metazoal kingdom.

Whilst food supply favourable Paramoecium multiplies by TRANSVERSE binary fission. Macronucleus divides into two by amitosis : micronucleus divides into two, however, by process which suggests, if it is not, true karyokinesis. Mouth, peristome, and body divide next into two : whole animal divides into two new Paramoeci.

If food supply unfavourable, as for example if its environment dries up, Paramoecium proceeds to encyst. Process of encystment of Ciliophera very curious. Thick, translucent cyst wall is secreted. Inside it the still motile ciliate continues to revolve, like a Catherine wheel, but ever more and more slowly. Food particles etc. are got rid of and endoplasm becomes very translucent. By degrees movements slow down, protozoon rounds up, becomes immobile. Contractions of contractile vacuoles often the last signs of movement to disappear. In this state the cyst remains, for weeks if necessary, in drying environment. When conditions again favourable cyst wall ruptures or dissolves, and ciliate swims actively out of it.

Thirdly, in addition to transverse binary fission and encystment, we have a **MOST EXTRAORDINARILY CURIOUS PROCESS OF CONJUGATION AMONGST THE CILIOPHORA.** It resembles nothing anywhere else in life. It consists of the interchange of nuclear elements between two different and apparently similar individuals, which both appear to be hermaphrodite. Essentially this process is intended to lead to the rejuvenescence of both individuals. Nuclear matter is exchanged between both individuals, which subsequently proceed to multiply by schizogony. There is probably no parallel anywhere in life to this most curious, and unique phenomenon : if there were the whole course of life upon the globe would have been changed. Imagine, for instance, if man were hermaphrodite and coitus simply meant interchange of sex chromatin between two similar individuals, which immediately afterwards went in for production of offspring by binary fission....Process appears to have no parallel, to represent a random attempt on the part of Nature to introduce an entirely new form of reproduction.

Ordinarily Paramoecium reproduces by transverse binary fission for about 600 or more generations. Its protoplasm then appears to be used up and some process for rejuvenescence necessary. Two apparently similar individuals come to lie side by side in their longitudinal axes, whilst still moving. Cell wall between them breaks down. In each the macronucleus simply goes to pieces and takes no part in conjugation. Micronucleus in each divides into two, then four. Of these quarters three break down, leaving in the cell only one-quarter of the original micronucleus. This now divides into two halves. Each half=⅛th of original micronucleus of individual, next passes over into the other and fuses with the similar chromatin in the other. New cell walls next formed between the two. Both separate. From the zygote single nucleus in each a new macronucleus is next formed : each becomes a new, complete Paramoecium. It is to be noted that subsequently, of the two conjugating individuals, one now commences to divide rapidly by transverse binary fission=? female : the other more slowly=? male. But whole process absolutely, unique in living forms, and has been subject of closest study, and, naturally of the wildest speculation.

Widest possible variation among free living ciliates in both morphology and life cycles, but in general are built upon same general basal plan. They are usually identified by their characteristic movements, dependent upon the position of cytostome and arrangement of their cilia. The parasitic ciliates form a large group, though very small in number as compared with the free living genera. Opalina ranarum of the intestine of the frog a good example. Has no cytostome, and belongs therefore to the Astomata. No contractile vacuole and has prominent myonemes, lying under pellicle, and causing parasite to bulge and shorten or elongate and get narrow.

Lives by osmosis. Dobell, in a striking memoir, has shewn that state of Opalina depends on state of its host : if frog be starved the Opalina degenerate, chromatin breaks up into irregular masses, big eosinophile granules form in cytoplasm, parasite breaks down. Opalina is multinucleate. During summer and autumn it multiplies in frog's rectum by transverse binary fission. In spring however the dividing forms get smaller and smaller, some remain in frog, others encyst and are passed out in faeces into water and come to infect the tadpoles of the next generation. Nyctotherus a very common parasite of frogs : especially in India a form which gives the appearance of a bit of rope swimming, species ?. The Gymnostomata are parasitic ciliates of the gut of ruminants, and have only a rudimentary cytostome.

In the human intestine one valid and three doubtful species claim attention :—

1. *Balantidium coli.* Discovered, 1856, by Malmsten in Stockholm, in two dysentery cases. Apparently identical organism found in gut of pigs. Ramsay in Assam finds apparently identical Balantidium in gut of sheep, and similar one described from monkeys. ? are these all one and the same, or different species, each specific to its own host ?

Is the largest protozoon of the human intestine. Roughly oval in shape, 50 to 70 μ long, 40 to 60 μ broad. Possibly races of it of different dimensions, as also with Paramoecium.

Balantidium infection especially prevalent in Philippines, but also world wide in distribution, and occurs in India, especially near Karachi, and in Assam.

Anterior pole more pointed than posterior. Body slightly asymmetrical. Peristome starts ar anterior end, leads as a funnel shaped depression down to cytostome situated just below the anterior pole. Surface on which peristome opens= ventral. Shape subject to squeezing, bulging, and distortion. Dorsal surface more convex than ventral.

The rows of cilia give the parasite appearance of being striated. Peristome leads down to cytostome, whence short oesophagus with undulating membrane leads down into endoplasm. Large macronucleus lies near middle of body, kidney or bean shaped, or—in side view—oval. Contains densely packed chromatin grains and a few larger nucleoli, bounded by very definite nuclear membrane. Micronucleus very small, spherical, lies in depression or bay on ventral aspect of macronucleus. Two contractile vacuoles, one near each pole, pulsate slowly and are difficult to see. At extreme hind end is a definite aperture in cell envelope, ? cell anus. Endoplasm contains food vacuoles, debris, leucocytes, R. B. Cs., quite often tissue fragments etc. The parasite is said to secrete a haemolysin, but no proteolytic ferment (contrast E. histolytica).

Lives in part of colon where contents fluid, ESPECIALLY CAECUM. Has been found in appendix. At times found deeply embedded in the gut wall. Multiplies by transverse binary fission. First micronucleus divides by mitosis, and then macronucleus divides by amitosis. Budding described by Walker, 1909, but ? ?. Conjugation described by Brumpt, 1913, and others, but no good account of process exists.

Infection acquired by swallowing the resistant cysts which are passed in the faeces of the Balantidium carrier. During encystation the parasite rounds up, secretes thick, translucent cyst wall with double contour. Revolves at first actively, then more and more slowly round and round inside cyst wall for some time. By degrees becomes motionless, cilia lost, parasite shrinks inside cyst wall. Cysts round or slightly oval, 50 to 60 μ diameter. Are the largest protozoal cysts met with in human faeces. Irregular refractile bodies often seen in fresh cyst, probably plastids, = food reserve and comparable to the chromatoid bars of E. histolytica cysts. The posterior contractile vacuole often continues to pulsate for some time after the parasite has become motionless. Rarely cysts may be encountered containing two individuals, ? encystment during conjugation, or ? binary fission within the cyst. The cysts will survive for weeks in faeces if not completely desiccated.

Balantidium coli occurs in both tropics and non-tropics, but is especially common in tropics and above all Philippines. Not yet grown in culture outside the body. Strong's Philippine statistics give a 30 % mortality for balantidial dysentery. In sections of ulcerated gut Balantidium found in intestinal follicles, in submucosa, in muscularis mucosae, even in the lymphatics. Whole question of association of Balantidium infection in man with Balantidium of the pig a very important one. Much work wanted at it.

Balantidium infection also present in pigs in Great Britain, but so far no human case reported from same locality. In balantidial dysentery there are localised ulcers in colon wall plus almost universal necrosis of the mucosa of the whole colon. Walker says the Balantidia mechanically displace the mucosa cells and creep into the submucosa where they lie in chains.

Haliwa and von Haus, 1920, record case of renal and ureter infection, but ? ? Marshall, 1911, described Balantidium coli from post mortem spleen of patient with kala azar, but ? ? was this an accidental free living ciliate. Hinkelmann described Balantidium in peripheral blood of human beings, but observation almost certainly wrong.

Balantidium infection usually quite harmless : 90 % of infections are present only as carrier state. But when it does cause disease it causes most severe and intractable dysentery. Brumpt, 1919, transmitted Balantidium from monkey to monkey by rectal passage, and also parasitised monkey with Balantidium coli from pig. Walker, 1913, infected 12 out of 13 monkeys with pig Balantidium, and 2 out of 4 monkeys with human Balantidium. Whole trend of evidence, says Dobell, is that Balantidium coli of man, pig, monkey are identical : thus Young and Walker, 1918, record a gut stripper in a factory who often got pig's faeces into his mouth, heavily infected with Balantidium coli.

Attempts to infect dogs, cats, kittens etc. so far negative results. In pigs the parasite usually produces no symptoms, but Brumpt gives evidence of lesions in pig's colon similar to those of balantidial dysentery in man. In monkey the parasite causes sometimes no symptoms, sometimes severe dysentery, as in man.

2. *Balantidium minutum.* Very doubtful parasite. Described by Jakoby and Schaudinn, 1899, in faeces of a German from N. Africa who suffered from alternating diarrhoea and constipation. 20 to 30 μ long by 14 to 20 μ broad. Buccal apparatus said to be more marked than in B. coli. Peristome is long and narrow and passes deeply down into centre of parasite, where cytostome is situated. Only one contractile vacuole, near posterior pole. Macronucleus spherical, 6 to 7 μ diameter, micronucleus tiny, only one μ diameter, placed anteriorly to macronucleus. Cysts usually oval, not round. Schaudinn also found it in case from Schulz. Brooks, 1908, records " Dr. Russell " as having found B. minutum in faeces of Porto Rico soldiers, but no reference or description : Sangiorgi and Ugdulena, 1917, found it in faeces of an Italian soldier and cultivated it in peptone water : (probably this was an accidental and coprozoic form and not a true parasite). Pinto records it in Brazil, in 5 out of 3917 stools examined. Does B. minutum really exist or not ? ? Confirmation wanted.

3. *Nyctotherus faba.* Described by Jakoby and Schaudinn from faeces of same patient as above, 1899. Organism bean shaped, flattened dorso-ventrally. 27 by 17 μ. Body clothed

with fine, short cilia: mouth with longer cilia=adoral zone. Peristome cuts out right upper quadrant of body, and from it depression runs to cytostome at centre of parasite, thence short, curved oesophagus into endoplasm. Single large contractile vacuole near posterior end, and cell anus. Macronucleus near centre of body, large, spherical, 6 to 7 μ diameter. Its chromatin arranged in 4 or 5 large blocks or masses, deposited on inner aspect of nuclear membrane. Micronucleus spherical or comma shaped, close to macronucleus. Said to form cysts.

This ciliate may possibly exist as a free living or coprozoic form, but very doubtful whether it is a true intestinal parasite of man. Still awaits any real confirmation.

Doubtful Ciliates. Here a wide field for speculation: very many free living ciliates from water used to wash bed pan etc. having been mistaken for true intestinal parasites Most of such forms only seen once and by one observer and all doubtful. Thus :—

Guiart, 1903, described a "Chilodon dentatus" from faeces of a Frenchwoman. Manson and Sambon, 1919, described Chilodon uncinatus from person just returned from N. Rhodesia. Chilodon, several species, a very common free living ciliate: e.g. very common in sewage effluents in Calcutta tanks, and these observations probably only free living forms. Schulz, 1890, reported Colopoda cucullus from faeces of a patient. Colopoda an extremely common free living ciliate.

Castellani, 1905, described Nyctotherus africanus from faeces of a Baganda native suffering from sleeping sickness. Hour glass shape, macro- and micro-nuclei, one contractile vacuole, 45 by 38 μ, covered with very fine cilia. Nucleus described as similar to that of N. faba, with characteristic arrangement of four or five chromatin masses on inner aspect of nuclear membrane. Said he found these parasites in large numbers deeply embedded in mucosa of caecum at post mortem examination. Dobell says he knows of no other ciliate with similar morphological peculiarities, and anyhow it is not Nyctotherus.

Krause, 1906, described ciliate from faeces of German case of typhoid. Oval, 90 to 400 μ long, 60 to 250 μ broad. His illustrations, according to Dobell, represent hopelessly badly fixed and stained parasites. Proposed name Balantidium coli giganteum, amended by Doflein to Neytotherus giganteus. Nature of this parasite doubtful, but again probably free living form. Martini, 1910, described tiny ciliate 35 by 18 μ, in three cases of dysentry from Tonkin. Appears to be identical with Cyclidium, a well known free living ciliate. Castellani 1914, describes new species, Entoplasma. Name bad anyhow and Dobell considers it a badly fixed and stained Balantidium. Marshall's Balantidium from the spleen and Hinkelmann's from peripheral blood probably merely free living ciliates from saline or distilled water used. Barlow, 1915, describes a Honduras variety of B. coli, 100 to 175 μ long, 79 by 100 μ broad, "nucleus" inconspicuous, micronucleus rarely to be made out, vacuole rarely present and when present does not contract. Dobell regards this as posssibly free living ciliate, certainly not Balantidium, and author's observations probably inexact. Sangiorgi, 1919, describes Albanese variety of B. coli found in wells of Valona, almost certainly merely a free living ciliate from the well water.

The *Acinetaria* or *Suctoria* are the other, second big subgroup of the Ciliophora. Mostly free living and mostly marine forms. Some are epizoic, attaching themselves to gill plates of fish and marine fauna. A few are entozoic. The young trophozoite is a free swimming form covered all over with cilia. The adult is sedentary, attaches itself to any floating object. Adults have lost cilia and mouth, but develop special tentacles for catching prey. Some of the Acinetaria acquire a tent or shell, either secreted by the protozoon, or consisting of an extraneous deposit of foreign sandy particles, lime salts etc. deposited in a gelatinous matrix. The group contains many very complex and very highly organised protozoa.

Practical Notes.

The way, par excellence, to study the Ciliophora is under dark ground illumination with low power objectives. The ciliary movements and adoral zones are admirably brought out. For staining occasionally good results may be obtained by killing over osmic acid vapour, completing fixation with methyl alcohol, thorough washing and then staining with one of the Romanowsky methods; have seen Opalina well stained by this method. In reality however the only reliable method is the Iron Haematoxylin one. Here there are special difficulties as the large bodies of the Ciliophora often take the stain very intensely and decolourisation may take some days in one per cent. iron alum solution : even stronger, 8 %, solutions may be required to decolourise. Different individuals decolourise at different rates, and whilst it is very difficult to get a uniformly stained slide, sometimes very pretty results may follow. After staining, the slide should be brought up through the graduated alcohols and then to alcohol-xylol, and xylol, and mounted, as the ciliates are too large for film preparations.

Literature.

Chapter 6 of Dobell and O'Connor's "Intestinal Protozoa of Man" contains an admirable account of the whole subject, beautifully illustrated.

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LECTURE 17.

THE SPIROCHAETES. RELAPSING FEVER SPIROCHAETES. S. MORSUS-MURIS.

Immense importance of the spirochaetes in medicine. Great discussion as to whether they are bacteria, or protozoa, or neither—but direct descendants of primitive protists, which have evolved along neither animal nor plant lines. Are long, narrow, thread-like forms with flexible cell membrane. No definite nucleus. No flagella or other organs of locomotion. Dobell, in a most careful and classical study of them, extending over years and including allied and similar forms, concludes :—1. Have no flagella or motile organs. 2. Definite yet flexible cell envelope. 3. Body is broken up by transverse partitions into almost square little compartments, like the structure of a bamboo, the septa being of very great fineness. 4. Most of them shew a strengthening axial filament of some sort. In the Cristispira this=the crista and is present as a prominent raised ridge along the concavity of the spirals. In other spirochaetes may be present as spiral like line along the longitudinal axis of the spirochaete. 5. There is no definite nucleus, on the other hand the chromatin is present throughout in the form of fine dots, situated in each chamber at the junction of the transverse septa with the cell envelope. 6. In many there are present metachromatic granules, (like those of a diphtheria bacillus), stain with Giemsa etc.

Spirochaetes are actively motile, though how they possess power of movement is a mystery similar to that of the movements of diatoms etc. They differ from the spirilla in that the spirochaetes have a flexible cell membrane and the spirals are not pre-formed : whereas spirilla have rigid structure and the spirals are pre-formed, like a corkscrew. Spirochaetes move :—1. backwards or forwards in their own longitudinal axis. Note that a spirochaete has no polarity, can move with either end in front. 2. bending, flexible movement of the whole body. 3. they may rotate like screw of a steamer as they move forwards or backwards. 4. they may bend laterally, some even may round till tip and tail touch, O and ring forms.

Many workers have claimed that spirochaetes are protozoa : basing their opinion upon Schaudinn's classical but erroneous 1904 paper, vide ante, Lecture 14. Schaudinn explained the absence of polarity in spirochaetes by saying that a spirochaete=two trypanosomes fused together by their ends. As well say, says Dobell, that man has no tail because he consists of two tailed animals fused together by their tail ends. Schaudinn's erroneous paper has led to different workers placing the spirochaetes among the Mastigophora as relatives of the trypanosomes, even among the Ciliophora on the ground that they must possess hypothetical cilia. Those who hold protozoal view emphasise in its favour :—1. spirochaetes, like parasitic protozoa, very difficult to culture on artifical media. 2. say they always divide by longitudinal binary fission, but this is very doubtful. 3. say the crista does not correspond to any bacterial structure.

On other hand evidence is all in favour of regarding them as true bacteria. 1. Their nuclear structure is like that of a bacillus. 2. they divide by transverse fission in at least most instances, as do bacteria. 3. their rate of multiplication is more like that of bacteria than of protozoa. 4. the serological and immunity reactions in spirochaetal infections resemble those of bacterial, not of protozoal, infections. Whole case for bacterial view predominant. Dobell classifies bacteria into 1. Haplobacteria, and 2. Trichobacteria, the latter of filamentous form. Haplobacteria are bacteria of varied form and include Coccidea, Bacilloidea, Spirilloidea and Spirochaetoidea : last the spirochaetes. Confusion due to 1. Schaudinn's erroneous lead, and 2. to workers using bad fixatives and worse stains and getting and studying distortion images.

Attempt to classify spirochaetes difficult. May accept the following genera or groups :—

1. Saprospira. Usually big forms free living with clearly chambered structure. 2. Cristispira, i.e. forms with a crista, parasitic in oysters. 3. Genus Spironema, (vel Spirochaeta =Spiroschaudinnia). 4. Treponema, not a true genus, but sub-genus : introduced by Schaudinn for spirochaetes of syphilis and yaws, characterised by very minute size and smooth, even symmetry of the coils. 5. Noguchi's genus of the Leptospira : which, in addition to usual spirochaete structure, have a spiral twist like the strands of a rope, plus a special tendency when moving to form curved loops at one or both ends. Accept this classification at least provisionally.

Innumerable free living spirochaetes known : e.g. Saprospira plicatilis of dirty pond water, S. gigantea of sea water, 60 or more μ long, S. anodonta. First spirochaete discovered was S. plicatilis in stagnant pond water by Ehrenberg, 1834. Sewage effluents abound in spirochaetes. Zuelzer has recently shewn, 1922, that in water there occur a series of spirochaetes which correspond morphologically with those that have been shewn to be pathogenic to man and animals : if their pathogenicity be proved this opens up a big field for enquiry.

Cristispira especially found in the crystalline style of oysters, e.g. S. balbianii, 100 to 150 μ long. Spirochaetes abound in the gut of animals and insects. Gut of almost every cockroach shews a very short, thick stumpy one : big one of similar pattern found in gut of S. African lion from Calcutta Zoo : also big ones seen in gut of rats and of frogs. The human spirochaetes are only a small section of a whole world of spirochaetes, and of other closely allied spiral organisms. The human ones however fall into the groups Spironema, Treponema and Leptospira.

Accounts of life history of spirochaetes very confused and much guess work, and inaccurate descriptions. The human spirochaetes live in blood, lymph or tissues of man. Great discussion as to true mode of division.

Dobell insists that it is always transverse, and is probably right. In relapsing fever spirochaetes at least it is undoubtedly transverse. On other hand transverse division preceded by spirochaete forming a loop on itself and hence appearance of longitudinal fission simulated. Noguchi and others insist that many spirochaetes divide by longitudinal fission. Yet if they did so, spirochaetes of any given species should vary in thickness. They dont : there are great variations in length, but thickness for any given species is usually very constant. The U appearance in division leads to great confusion.

Spirochaete life cycles perhaps more subject to wild speculation than those of any other parasites. In both man and transmitting insects they are supposed to have a granule phase. Inside cell membrane spirochaete said to break up into tiny infective granules. These are shed and accumulate in the internal viscera in man or in the coelom and Malpighian tubules and ova of ticks. Thus in S. gallinarum of fowls, a very favourite parasite for laboratory study, Balfour describes phases when no spirochaetes can be seen in blood films, but in the cytoplasm of the nucleated R. B. Cs. are big, deeply staining chromatin dots. These are supposed to be able to grow up again in spirochaetes. The dots are there all right, but whether they have anything to do with the spirochaetes is doubtful.

Many spirochaete infections transmitted by ticks and lice. In the tick the spirochaetes get through the gut wall into coelomic cavity and penetrate into every tissue of the tick, breaking down into tiny infective granules as they do so. Granules accumulate in Malpighian tubules, so that tick's faeces are infective. Tick itself is also infective if crushed into skin abrasion. Also its ova infected and so the next tick generation. Salivary glands of tick not infective if it be kept at 22°C, but if it be kept at 37°C they become infected and infective. In hereditarily infected young tick the granules accumulate around the developing Malpighian tubules and so infect new tick's body and faeces. Noller even claims that third tick generation may be hereditarily infected from second. Note that spirochaete infection may be hereditarily transmitted :—1. in man, as with syphilis. 2. in insects, as with relapsing fever ticks. In man mother usually infected from father and child next infected. But exact mechanism of hereditary transmission of syphilis has still to be worked out. Acton suggests two possibilities :—1. transmission direct from male parent to offspring, the spirochaetes being in the spermatozoon : with or without infection of the mother. This would account for the still births of syphilis, the foetus does not mature. 2. indirect transmission from father first to mother and thence across placenta to developing foetus, would account for live births of children with hereditary stigmata.

Chief contributors to spirochaete life cycles are Balfour, Hindle, Fantham, Leishman and others. Leishman first described granule phase in tick. Fantham says he has seen S. bronchialis shedding granules under dark ground illumination, but dark ground examination very open to errors.

Practically all human spirochaetes have now been cultivated on artificial media. The blood inhabiting spirochaetes readily cultivated on almost any medium, e.g. N. N. N. or Row's.

Wenyon's medium for spirochaetes as follows :—To 270 c.c. of 0.85% NaCl solution, pH=7.6, add 30 c.c. of 2% bacteriological agar, pH=7.6 Tubes are filled three quarters full and autoclaved at 120°C for 20 minutes. Cool on water bath to 50°C, and then carefully add 20 drops of fresh rabbit blood to each tube. Wenyon recommends obtaining blood by puncture of ear vein, with special box to hold rabbit, but in Calcutta at least this means sepsis and cardiac puncture is better. The tubes are not shaken but are incubated at 37°C for 24 hours before inoculation. Specially recommended for Leptospira. (Also see later under Treponema).

Disregarding systematic classsification of spirochaetes we may classify them for convenience as follows :—

1. The blood inhabiting spirochaetes, viz. those of the relapsing fevers and S. morsus-muris, the parasite of rat bite fever.

2. and closely allied to 1. The Leptospira group. L. ictero-haemorrhagiae, the cause of acute epidemic haemorrhagic jaundice, and L. icteroides, the cause of yellow fever: with possibly others.

3. The Treponema group, T. pallidum of syphilis and T. pertenue of yaws.

4. Vincent's spirochaete, which, together the fusiform bacillus is responsible for Vincent's angina, Naga sore and tropical ulcer. Groups 1 to 4 are all pathogenic.

5. The harmless spirochaetes of skin and mucous membranes : such as S. refringens, S. aboriginalis found in inguinal granuloma, S. acuminata and S. obtusa in yaws lesions, S. gracile of the genitalia, S. pseudopallida in carcinoma lesions, S. microdentium, S. mucosum of the mouth, S. minuta of the nose, and S. bronchialis, met with in Castellani's bronchitis, and possibly pathogenic.

6. The intestinal spirochaetes, chiefly S. eugyratum, S. vincenti, S. stenogyratum, S. refringens etc.

This list not in any way exclusive but merely mentions the chief ones.

Relapsing Fever Spirochaetes.

Considerable discussion here as to whether only one species or several. Todd, 1922, in Byam's "Practice of Medicine in the Tropics" considers that there is only one species, Spironema recurrentis, and others are mere varieties. Others describe the following as different species :—

1. S. recurrentis vel S. obermeieri, of European relapsing fever. Short forms 7 to 9 μ, long ones 16 to 19 μ. Transmitted by pediculi, and also possibly by bed bugs. Nuttall experimentally transmitted the infection from mouse to mouse by bed bug bites. Karlinski found bed bugs from the house of a relapsing fever patient infected under natural conditions.

2. S. duttoni of African relapsing or tick fever. 16 to 24 μ long, and said to be more variable in length than 1. Ends

pointed ? Transmitted by tick, Ornithodorus moubata. Dutton and Todd proved experimental transmission on monkeys : found tick's salivary glands not infective if kept at 22°C, but become infective if tick kept at 37°C. Same species also occurs in Columbia in C. America and transmitted by tick, Argas americanus.

3. S. noyvi, cause of N. American relapsing fever. 17 to 20 μ.

4. S. carteri, cause of Indian relapsing fever. Louse transmission proved.

5. S. berbera, cause of relapsing fever of N. Africa. Over 12 μ long with irregular open spirals. Monkeys susceptible, mice and rats only with difficulty.

Those workers who accept diversity of species base their claim on 1. minor differences in length, average number of coils, morphology etc. of the spirochaetes concerned. 2. differences in severity of the symptoms produced by different strains. 3. different susceptibility of different laboratory animals to experimental infection. 4. serum of animal immunised to one strain neither agglutinates nor destroys other strains. Animal immunised to one species still susceptible to infection with the others. 5. different transmitting agents. Todd however is great authority on the disease and admits only one species.

Relapsing fever spirochaete first discovered by Obermeyer, 1873. Vandyke Carter first found parasite in Indian cases, inoculated himself with blood from patients and took the infection, thus proving blood to be infective. Ross and Milne, 1904, and Dutton and Todd, 1904, found African spirochaete in relapsing fever cases. Proved Ornithodorus moubata to be transmitting tick by experiments on monkeys. Mackie, 1907, the Sergent brothers and Nicolle, 1912, and Cragg, 1922 proved louse transmission of relapsing fevers and also hereditary transmission to next pediculus generation. Relapsing fevers fall into two main groups : 1. louse transmission via faeces, via louse crushed into skin, and via louse progeny. 2. tick transmission via faeces, via tick crushed into abraded skin, via progeny, and also via bites and salivary infection.

Accidental infection in laboratories has often occurred. The spirochaetes will penetrate through any trivial abrasion, or under nails, through unabraded mucous membranes, and even —possibly—through healthy skin. Cragg's 1922 paper a classic, which should be read by all Indian students. Greater prevalence of the fever among males in the U. P. due to their habit of crushing infected lice between finger and thumb nail, the spirochaetes entering the soft nail bed. Special association of relapsing fever epidemics with years of famine and spring months in U. P. explained as follows. Population more susceptible than usual and poorly nourished. If now we get in the spring the temperature remaining unusually low and unusually high humidity with delayed onset of the hot weather, then the annual delousing which usually takes place at the onset of the hot weather does not occur, the pediculi live longer, and epidemic relapsing fever sets in. Both ticks and lice in relapsing fever infective if crushed into skin abrasion or even into unabraded mucous membrane. Coxal secretion from Malpighian tubes infective. S. gallinarum of fowls transmitted by tick bites, also by tick faeces and tick progeny.

Mackie in India shewed special association of lice with Indian relapsing fever. Of 170 verminous boys at a school 137 became infected during epidemic : of 114 girls in adjacent school, who were much less verminous, only 33 became infected. Nicolle proved louse bite not infective : fed 4,707 lice on case infected with S. recurrentis : and subsequently fed them all on a human volunteer,—protecting from faecal contamination by only allowing lice to bite through gauze : volunteer did not take. Louse becomes infective on 6th day after feed. Lice fed on patient during afebrile period, when no spirochaetes can be detected in the blood, also become infective. ? unrecognised granule phase of spirochaetes in blood during afebrile intervals. Cragg proved louse cycle in India for S. carteri.

Taking relapsing fever spirochaetes generally their length varies from 12 to 36 μ, but sometimes as short as 6 μ, and as long as 55 μ, whilst, during convalescence, enormously long forms up to 100 μ, and more have been described, ? due to end to end agglutination of spirochaetes. Width in Leishman stained films may appear to vary, usually about 0.3 μ, but the width of spirochaete species does not appreciably vary if proper fixatives and stains be employed. Ends tapering. NO flagella found in any spirochaete if properly fixed and stained, (Dobell). On cross section spirochaetes are circular and not band like. S. recurrentis does not corkscrew, but bends. Waves usually about 4 μ from crest to crest, and 0.8 μ deep, but in a tightly wound specimen may only measure 2.2 μ from crest to crest. Often seen coiled in wreaths and loops and irregular patterns. In fresh preparations actively motile. Occasional forms seen as if ? attached to R. B. C. or leucocyte. Divide undoubtedly by transverse fission. Todd says longitudinal fission also occurs. (? ?). On Giemsa staining may shew metachromatic granules. Breinl, for S. duttoni, describes how in internal viscera during the afebrile phases the spirochaetes enter into endothelial cells, (? or are phagocytosed by them ?), coil up, encyst inside delicate cell membrane within the cytoplasm of endothelial cell, break down into granules : then cyst ruptures and granules set free, grow into spirochaetes. Cycle badly needs confirmation if true.

Spirochaetes found in peripheral blood only during febrile periods, not in afebrile stages. Some workers describe agglutination of the spirochaetes together at period of crisis in temperature chart, and spirochaeticidal crises, resembling those of trypanosome infections. Hindle considers that whilst spirochaetes absent from peripheral blood there is going on multiplication schizogony cycle in internal viscera via granule phase. The relapsing fever spirochaetes will pass through the placenta from mother to foetus, and infect the latter.

In fed ticks, when examined some days after feeding, some of the spirochaetes are normal, others have clubbed ends, some are short, others long. Chromatin appearances bizarre, with granules ? chromatin, ? metachromatic. Nature of and possibility of granule phase in tick much discussed question. Spirochaetes said to produce two kinds of granules, 1. large ones which are extruded as terminal or lateral swellings, and 2. small ones which are formed within the cell envelope, like spores of bacteria, and are said to be capable of growing up into new spirochaetes when shed. But all work on spirochaetes with exception of Dobell's done with dry fixation etc., and imperfect technique and appearances open to doubt. In Malpighian tubes of tick and around ova are seen short 8 μ spirochaetes, granules, comma shaped forms, coiled and uncoiled spirochaetes, and ? encysted spirochaetes. The tick tissues shew no reaction to the invasion. In classical set of serial sections Wolbach has shewn existence of spirochaetes throughout tissues of the infected tick. Tick retains infectivity for at least five weeks, possibly longer.

In louse transmission matters are somewhat different. Within a few hours of feeding NO spirochaetes can be found in the gut and the louse is not infective. About 6th to 8th day after feed spirochaetes appear in the gut and in the tissues of the louse and are small, suggesting that they have developed from a granule phase. Louse now becomes infective.

To sum up :—1. number of species of relapsing fever spirochaetes a question at issue. 2. two well defined groups : tick and bug group include fevers of Africa, N. America ; louse group fevers of Europe, India, Asia, N. Africa, Manchuria,

3. In tick and bed bug group tick is infective all the time and via bite, faeces, coxal fluid, progeny and crushing of infected tick. 4. In louse group definite cycle of some sort with louse not infective till some days after feeding and no salivary transmission. 5. spirochaete multiplication, both in viscera of man and in tissues of insect, through a granule phase is probable, although not yet fully proved.

Some spirochaetes can pass through Berkfeld filters V,N,W.? in a granule phase, or ? simply owing to ther flexibility.

Relapsing fever spirochaetes fairly easy to cultivate. Row's medium. Wenyon's medium as above. Kliger and Robertson, 1922, recommend the following :—The basis of the medium is either ascitic fluid or one part of rabbit or horse serum to two of saline. To each 10 c.c. of this add one c.c. of a 10% peptone broth : which makes the final concentration of peptone one per cent. Adjust reaction to pH 7.2 and distribute into one cm. wide tubes in 3 to 4 c.c. volumes. Inoculate tubes with a drop of the infected blood, or for subculture with a drop of original culture. Cover with 1½cm. layer, of oil not thicker, (as spirochaete is a strict aerobe). Incubate at 28 to 30°C. The peptone acts as a buffer and optimum growth is obtained at a pH of 7.2 to 7.4. Subculture weekly.

All three species of human lice carry the infection. So also do lice etc. of infected monkeys. Nicolle records case where man had over 15,000 louse bites from infected lice, but failed to take : yet when a single infected louse crushed and placed on his conjunctiva infection took. Manson and Thornton believe tick transmits to third as well as to second generation. In E. African epidemic they found from 30 to 50% of local ticks infected, and ticks retained infection for 18 months.

Immunity phenomena in relapsing fevers. Immunity varies. Some African villagers get two attacks in same winter. Immune serum possesses powers of spirochaete lysis, agglutination, precipitation, and also shews complement fixation to specific spirochaete antigen, prepared from spirochaete cultures, freed from R. B. Cs. If drop of blood from convalescent and immune patient be mixed with drop of blood from acute case and the preparation ringed with vaseline and incubated at 37°C, the spirochaetes become first clumped, then immobilised, then gradually destroyed. The immunity resulting from infection, whether natural or experimental in the laboratory, is only against the particular species of spirochaete concerned, and not against the others.

In addition to being found in the blood, the relapsing fever spirochaetes also found in the C. S. fluid, sweat, tears, sputum.

Dark ground examination is the best way to find them. Failing this take thick blood films, and de-haemoglobinise and stain. Can be stained by almost any aniline dye, stain well by Leishman or Giemsa's stain, or even with watery carbol fuchsine. For test in experimental animal take at least 20 c.c. of patient's blood into citrated saline. Inject into peritoneal cavity of young white rat or monkey. Examine blood daily for the next ten days.

S. morsus-muris.

Rat bite fever is a true relapsing fever, due to S. morsus-muris. About 80 cases so far reported from Japan, America, England, France, Italy, Scotland, Spain, Germany. About 3 % of house rats examined are said to harbour the spirochaete. Cat bite fever of Japan, and weasel bite fever aparently identical : ? ? do the cats and weasels get the infection from eating infected rats.

In India at least rat bite fever always due to S. morsus-muris. Row, 1917, found it common in Bombay, and so does Permanand, who has recently kindly supplied the School with a strain. Found in Shillong in one case by Knowles, 1918, and in another by B. M. Das Gupta, 1922, and strain brought to Calcutta and kept going for six months in white mice. Between 1920-22 Knowles saw three cases in Calcutta with specific spirochaete present in lesions. Stewart, 1922, found this spirochaete in rats in Kidderpore docks. Disease, which must be carefully distinguished from fever merely due to sepsis following rat bite, is quite common in India, if it is looked for. In Japan even more so.

Schottmuller, 1914, and Blake, incriminate Streptothrix muris ratti as cause of rat bite fever. Whether this is so or not in America dont know, but it does not apply to India. Secondary streptothrix infection of any ulcer very common in the tropics, and may be merely a secondary invasion. The true parasite cause in India is S. morsus-muris.

S. morsus-muris first isolated by Japanese workers, on examining site of the bite in 1916, by Futaki. Patient usually bitten at night, and there follows incubation period of 5 to 90 days, average 2 to 3 weeks. Bite, which has in the meantime—usually—healed up completely, now becomes swollen and painful. Erythematous rash occurs, sometimes missed. Fever next appears and is of typical relapsing type, with the spirochaetes in blood. Two types of the spirochaete : in puncture of the site of the bite long forms, 6 to 8 μ, with 4 to 8 turns :

in enlarged and swollen lymph glands draining site of bite and in the blood stream in very scanty numbers short, stumpy little spirochaete, thick at middle, pointed at ends, 3 to 5 μ long, AND A VERY SHORT SPIROCHAETE WITH ONLY TWO AND A HALF OR AT MOST THREE TURNS. That the short forms in the blood and the long ones at the site of bite identical proved by culture, when both yielded identical strains.

Animals susceptible are monkeys, guinea pigs, white rats and especially white mice. Strain kept alive at Calcutta by B. M. Das Gupta for six months by repeated passages in white mice : in inoculating passage mouse it should receive both blood and ground up spleen emulsion of previous mouse. Incubation period in experimental animals about 10 to 11 days. In animals a similar relapsing type of fever may even set in occasionally. Spirochaetes in man found in all internal viscera including the kidneys. Guinea pigs experimentally infected also by letting infected rats bite them.

Spirochaete in blood in man later clears. Are sometimes found in urine and have been reported from saliva. Bass states, and several others emphasise, that S. morsus-muris is quite different from other spirochaetes. It is very highly motile, is said to have a rigid and not flexible structure, and to have a single flagellum at each end. If so, although personally have never seen the flagellum in examining dozens of Giemsa stained slides, this organism will have to go out of the spirochaetes and be reclassified among the spirilla. On other hand its association with a relapsing type of fever suggests a true spirochaete.

Rat bite fever especially among agricultural labourers and sailors. Interesting Calcutta cases, one seen Oct. 1920, two Nov. 1921. Latter were, father and daughter both bitten at night by—presumably—the same rat. Both infected with true rat bite fever with spirochaete in lesions. The spirochaete is NOT in the rat's saliva, but is in its blood and tissues. Rat probably only infects man if its gums are abraded. Popular idea that only female rats are infective and then only during breeding season. Always be on the look out for S. morsus-muris in India.

Practical Notes.

The blood inhabiting spirochaetes can readily be studied in either thick or thin blood films. For thin films the readiest stains are either Leishman's or Giemsa's. Thick films should be dehaemoglobinised and stained as before. For S. morsus-muris either the site of the bite, after cleaning with a swab soaked in spirit and drying, should be punctured with a capillary pipette, or gland puncture done with a hypodermic syringe or blood inoculated into a white mouse.

Indian ink method for spirochaetes. Burri's. Take a perfectly clean glass slide. Near one end place a drop of the fluid to be examined, blood, or juice from chancre e.g. Beside it place a larger drop of Indian ink. The ink used must be of good quality: many of the Indian inks on the market are useless. Chin Chin or Gunther's best. Mix the two drops and with a spreader carefully spread out as a film of uniform thickness from one end of the slide to the other. Allow to *completely* dry, which takes an hour. Examine with the oil immersion lens: the unstained spirochaetes stand out well against the black-brown background.

Harrison's collargol method. Put one part of collargol into a bottle with 19 parts of distilled water. Shake repeatedly to ensure solution. At one end of a clean slide place a drop of the material to be examined: add a similar sized drop of the collargol solution. Mix and spread into film. Dry without blotting. When throuoghly dry examine with oil immersion lens. The silver deposits into the spirochaetes, oxidises and turns black, so that they stand out black against a colourless background.

Benian's Congo Red Method. Thoroughly mix on slide one or two loopfuls of 2% aqueous Congo red with one loopful of blood, serous exudate or suspected material. Spread into even, thin film. Allow to dry completely in air. Wash with 1% aqueous HCl very gently. Do not use water or blot. Drain off acid and dry in air. The spirochaetes appear white against a uniform blue background.

Dark Ground Illumination.

The special method of examination for spirochaetes is the use of the dark ground apparatus, which is invaluable in all protozoological work, for examining flagellates, ciliates, etc. as well as for spirochaete work. The usual form of apparatus is Zeiss' Paraboloid condenser, which consists of (a) the silvered condenser, (b) a funnel stop for use with the oil immersion lens, and (c) a gauge with slits 1.1 and 1.2 mm. thickness for gauging the slides to be used.

1. The source of light must be a brilliant one: e.g. a Pointolite, electric arc, acetylene bicycle lamp, incandescent

gas or petrol vapour lamp or other. In some recent patterns of dark ground condenser the illuminator is an electric lamp inbuilt with the condenser in one single piece of apparatus.

2. In front of the lamp must be placed a condenser to focus the rays of light from the lamp so that they just fill and no more the whole of the concave mirror of the microscope. A bull's eye condenser may be used, but one of the simplest forms is a round flask filled with distilled water tinged green with copper sulphate crystals. This acts as a condenser, gives monochromatic light, and cuts out the heat rays. Testing with a bit of white cardboard over the mirror so arrange matters that the whole of the concave mirror is filled with bright, uniform light. The microscope, if not already centred, must be accurately centred before dark ground illumination is used.

3. Rack down the substage and remove the Abbé condenser. For it substitute the dark ground condenser. The student should fully understand the principle of this. The condenser is silvered over except at its margins. Holding it up to the light tilt it about and examine from the upper surface : it will be seen that the field is dark except for a very narrow rim around. When the pencil of rays from the mirror strikes this silvered condenser, all rays are reflected back, with the exception of the rays entering the periphery of the condenser. These are transmitted and come to a focus on the slide. Hence any object lying on the slide is brilliantly illuminated by these oblique rays, but stands out against a black, dark background.

4. Unscrew the oil immersion 1-12th and into it drop the funnel stop. This cuts out peripheral rays and renders definition better. The 1-12th cannot be used without it.

5. Select only such glass slides as will fit the gauge, i.e. 1.1 or 1.2 mm. thickness. The cover slips must be the thinnest available, No 00 or No. 0. No 1 ruins the definition.

6. Having cleaned the slides and cover slips place the material to be examined, e.g. serous exudate from chancre, blood from relapsing fever case etc. on the slide. Use only a very small drop, cover with cover slip, and if examination is likely to be prolonged ring with vaseline. The utmost care must be taken that everything is scrupulously clean and free from dust, which ruins any dark ground work.

7. Rack up the condenser right up to stage. On top of it place a large drop of cedar wood oil. The oil must be thin and every care taken that no air bubble gets in. Holding the slide preparation upside down, on its under surface place a second

drop of cedar wood oil, free from air bubbles. Reinvert and gently lower on to the stage : so that there is a thin uniform layer of oil between the condenser and the under surface of the slide. Or distilled water may be used in place of oil.

8. Everything being now ready, first examine the preparation with the ⅔rds and see that the illumination is maximal and uniform and critical. If necessary move the flask or the lamp etc. until absolutely critical illumination has been secured. This is the whole secret of dark ground work. The ⅛th and oil immersion 1-12th can now be used in turn to examine the preparation.

In all dark ground work, as also in all work on fresh material, e.g. examination of stools for motile protozoa, the use of a Zeiss's binocular eyepiece, e.g. the binocular prismatic fitting for a monocular tube, Zeiss No. 829, is very helpful. Spirochaetes stand out as motile, shimmering threads, silvery white against the black dark background. Dark ground illumination of fresh material in searching for scanty spirochaetes is worth a dozen of stained material preparations.

In dark ground work the student may at first experience some difficulty in getting the right focus. Focus down on to any prominent objects present, e.g. R. B. Cs., and the right focus will be obtained.

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LECTURE 18.

LEPTOSPIRA. L. ICTEROHAEMORRHAGIÆ, L. ICTEROIDES, L. HEBDOMADALIS. FEVERS OF DOUBTFUL ETIOLOGY. RICKETTSIA.

L. icterohaemorrhagiae is the cause of Weil's disease, or epidemic acute haemorrhagic jaundice. This a disease of armies, very prevalent in 1800 in Napoleon's army in Egypt, in American civil war and of great importance in the Great war. Fever with sudden onset and 30% death rate. Spirochaete discovered by Inada and colleagues, 1915. Spirochaete found in blood in scanty numbers from 4th to 9th day of disease. Always present in urine. Urine loaded with albumin, casts, may be bile pigmented and even haematuria. Spirochaetes 6 to 9 μ long, may reach 20 μ, both long and short forms. In addition to usual spirochaete structure leptospirae have a spiral twist in their length and a special tendency to forms loops at the ends. When loop formed at both ends simultaneously they stop movement until the loop opens out again. Shew prominent refractile granules inside under dark ground.

Disease almost certainly exists in India. Parmanand, 1922, describes seven cases in Bombay, with spirochaete in urine. Excellent account and discussion opened by Glen Liston at Indian Science Congress, 1922, see I. M. G., July 1922, p. 268. Epidemic jaundice of Andamans, which only occurs among convict gangs working in fields probably spirochaetal. Whole of work on disease is war and post-war work. To find the spirochaetes in the blood use thick films, they are very scanty. Or inoculate patient's blood intraperitoneally into guinea pig. After 6 days' incubation period guinea pig goes down with disease similar to that in man, and the spirochaetes can be recovered from their blood and urine. To discover the spirochaetes which are scanty in the urine:—Take 20 c.c. of urine and centrifuge. Throw away supernatant fluid, add further 20 c.c. urine in same tube, and again centrifuge. Repeat until in all from 60 to 100 c.c. have been centrifuged in the same tube.

The deposit at the third or subsequent centrifuging will shew the spirochaetes.

L. icterohaemorrhagiæ readily cultivated. Kaneko, 1921, sums up methods of cultivation as follows :—1. Human ascitic fluid gives good growth if a little blood be added. 2. Various sera of animals successful, especially rabbit's. 3. Rabbit serum plus 2 to 5 parts of Ringer's fluid gives good results. (Ringer's solution is NaCl, 0.6 : KCl, 0.0075 : $CaCl_2$ 0.01—dried— : $NaHCO_3$, 0.1 gm. each : distilled water 100 c.c. Dissolve the $NaHCO_3$ before adding the $CaCl_2$.) 4. Haemoglobin is necessary for culture. 5. The addition of agar or gelatine favours growth. 6. A layer of paraffin on the surface of the culture helps. 7. Slight alkalinity best. 8. Most rapid growth takes places at body temperature, but once growth obtained the tubes can be kept at laboratory temperature. Recommend the following two media :—(a). Liquid medium. To 2 or 5 c.c. of Ringer's fluid at 45°C on water bath, add one c.c. of rabbit blood. Inoculate and cover with layer of sterile paraffin. (b). Semi-solid medium. To above fluid add 0.3 % ordinary agar. Or to 100 c.c. Ringer's solution add and dissolve 3 gms. agar, and add 9 c.c. of serum. Cover with paraffin after inoculating. Matsuzaki recommends simple blood agar.

Curious etiology of Weil's disease. Guinea pigs experimentally infected by feeding on material contaminated with blood or urine of case. Also by subcutaneous injection of blood or urine from patient. In Japan where disease was first discovered and is very prevalent, wild rats in large percentage shew similar, probably identical spirochaete, especially in kidneys and urine. Examination of rats all over world and especially in sea ports, such as Marseilles, shews similar findings. Believed that during the war disease was contracted in rat infested trenches by eating food contaminated with rat urine, rat blood or rat's bodies. Or soldiers' feet abraded and rat urine, or blood or dead bodies of rats in trenches infected abrasions.

Always be on the look out for Weil's disease in India.

Leptospira icteroides. Noguchi, 1918, may now be accepted as the proved cause of yellow fever. Noguchi's series of papers will be found in the Journal of Experimental Medicine, from Vol. 29, 1919 onwards to current date, 1923.

Noguchi started work, 1918, in Guayaquil, on 172 cases of yellow fever. 8 out of 74 guniea pigs inoculated with blood of patients got yellow fever symptoms and died with haemorrhagic jaundice. Their blood, liver films, kidney films filled with spirochaete, L. icteroides. Passaged from these to fresh guinea pigs, and to dogs, monkeys, etc. with again similar spirochaete findings and symptoms as before. Incubation period in experimental animals 72 to 96 hours. Most of infected guinea pigs did not die, however, but recovered and were then immune. Sera of convalescent yellow fever patients and of recovered guinea pigs shew immunity and agglutination phenomena to L. icteroides.

Next succeeded in getting pure culture of spirochaete from blood of yellow fever cases. Out of 11 consecutive unselected cases 3 yielded cultures of L. icteroides. At post mortem on cases found the spirochate in tissues and kidneys, also during life in urine. Next found wild animals of yellow fever districts infected with similar spirochaete, 67% of local wild rats shewed it. Guinea pigs inoculated with spirochaetes of these wild rats proved subsequently refractory to L. icteroides from human cases and cultures.

Turning to Stegomyia, the proved anopheline carrier, proved that virus can be experimentally carried by it from man to guinea pig, and from guinea pig to guinea pig by bites. Incubation period 8 to 12 days. Went on to serological reactions. L. icteroides very similar to L. icterohaemorrhagiæ, but serological reactions different. Noguchi concludes that, although the two diseases and two spirochaetes very similar, yet are not identical. Next produced very high titre antiserum against L. icteroides. 1 c.c. of 1 in 10,000 dilution protected guinea pigs against 5000 M. L. Ds. of infected guinea pig kidney emulsion.

From Guayaquil went on to Yucatan and there worked with similar results. Spirochaetes from Yucatan cases and animals gave all the reactions of the Guayaquil strains and agglutinated with Guayaquil sera. Went on to N. Peru. Similar results again. Recommends simple diluted rabbit serum as culture medium for parasite. Then commenced work on the toxicology of the disease : shewed that both L. icterohaemorrhagiæ and L. icteroides in culture produce toxins which cause slowing of heart beat or even complete heart block. Work still in continuation.

Noguchi's work much criticised : chief criticism is that the spirochaetes of these wild rats may have nothing to do with human spirochaetes : but it is up to the critics to carry out experiments to disprove, not to sit in armchairs and criticise.

Stegomyia the proved carrier of yellow fever. Possibility once believed to be serious of introduction of yellow fever into India with opening of the Panama Canal : as infected Stegomyia could then travel in direct tropical belt from Panama to India. Col. James sent on deputation by the Government of India. Original foci of the disease in Central America however are now being so thoroughly dealt with that danger of exportation of disease more apparent than real. Also ? ? have we already got yellow fever in India. ?

Classical work of the Yellow Fever Commission at Camp Lazear, 1900, Reed, Carroll, Agramonte, and Lazear. Mosquito proof hut divided into two portions by mosquito gauze screen. Into A put mosquitoes fed on yellow fever patients, into B clothing, bedding etc. from yellow fever patients, soiled with dejecta and urine, but mosquitoes excluded. Human volunteers slept in A and in B. Those in B remained well, those in A contracted yellow fever, and Lazear himself died of it, after bite of infected mosquito. Mosquitoes only take the infection from case during first 3 or 4 days' illness : remain infective 57 days or longer, i.e. long enough to reach India by direct boat from Panama. May possibly indeed transmit to next Stegomyia generation. Apparently no mosquito other than S. calopus can carry the infection. Serum of convalescent patients protects individual and helps in treatment of the disease. Blood of patients taken during first 4 or 5 days' illness, and heated to 55°C provides an antigen which has been used to inoculate against the disease. Exact mechanism of mosquito cycle remains to be worked out. Must be some developmental cycle as the mosquito only becomes infective 12 to 14 days after feed.

Fevers of Doubtful Etiology.

At this point we come to a whole group of fevers of doubtful etiology. Above we have two well defined groups of spirochaetal fevers, the relapsing fever group and the leptospira group. Allied are whole group of fevers where transmitting insect agents are known, and are either ticks, bugs, mosquitoes or pediculi, but where actual causative micro-organism is not known or where it has not yet been proved to be causal agent. Some of these diseases almost certainly spirochaetal, some almost certainly not. Curious how in Ross's day in India it was much easier to discover parasite of malaria than its transmitting host : but how today easier to find transmitting agent than nature of virus of the disease. The more important of these fevers are :—

1. *Dengue.* Discovery by Couvy, 1921, in epidemic that affected nearly all inhabitants of Beyrout, Syria, of short,

slender spirochaetes with two or three turns, and pointed ends in blood of patients the day before they shewed dengue symptoms. Later seen during first 36 hours of fever. Dengue probably a spirochaetal disease. Transmitted by mosquitoes, but transmitting mosquito may vary in different countries. Thus Ashburn and Craig, 1907 proved mosquito carriage. Cleland, Bradley and McDonald proved Stegomyia transmission for Australia and S. Sea Islands, 1916. Lalor, 1913, suggested Stegomyia transmission in Rangoon of similar seven day fever of Indian ports. Culex fatigans also strongly suspected, and is possible vector in epidemics in India up country. Koizumi, Yamaguchi and Tonomura, 1917 give classical study of disease, 6000 cases with only one death. Constant leucopaenia present. No blood parasites seen. Injection of de-fibrinated blood from patient gave negative results with dogs, rabbits, white mice, but guinea pigs took and died in from 7 to 36 days, and their blood infective to other guinea pigs, no blood parasites seen. Cleland and Bradley passaged the disease by artifical inoculation through four generations, and found virus present in blood 18 to 90 hours after onset of symptoms : as small a quantity as 0.00005 mil. of blood infective from man to man. In Formosa Japanese incriminated S. calopus but not Culex.

2. *Seven Day Fever* of Indian ports probably=dengue. Seven day fever of Japanese ports possibly a different disease. Discovery by Ido, Ito and Wani, 1918, of a spirochaete, Leptospira hebdomalis in blood of cases, and also of field mice.

3. *Sandfly fever*, is probably identical with three day fever of the tropics and sub-tropics. The chief carrier is Phlebotomus papatasii, but P. minutus, P. perniciosus and P. major may also transmit. The virus is a filter passer and Whittingham 1922, isolated a leptospira as the causal agent. Couvy, 1921, also claims to have found a spirochaete in the blood in sandfly fever cases, 3 hours and 24 hours after the onset of symptoms.

In 1 and 2 and in closely allied fevers we probably have a group of fevers due to one and the same spirochaete, described under different names in different localities. At some stage these spirochaetes or this spirochaete is a filter passer, and the transmission is by mosquitoes, chiefly Stegomyia.

4. *Japanese River Fever, or Tsutsugamushi disease.* An acute febrile disease prevalent in certain parts of Japan, Formosa, and China, caused by the bite of a mite, Leptus or Trombidium akamushi. The mite is probably distributed by a

small field-warbler bird, Acrocephalus orientale. Occurs especially during the harvesting seasons in certain Japanese valleys when the labourers are in the fields and get bitten. Nagayo found piroplasma-like forms in the spleen and lymph glands. Kawamuro and colleagues failed to find any parasites, but proved the blood to be infective. Even as little as 0.001 c.c. may infect monkey, although more usually 0.1 c.c. required. Virus also present in enlarged lymph glands. Heating the blood to 50°C for an hour destroys it. Hayashi, 1917, describes the parasite as minute rods, spherical or ring shaped and occurring in lymph glands, spleen endothelial cells and endothelial cells of lymph nodes, also free in the blood, and very rarely in the R. B. Cs. Appears to have vegetative and generative cycles. Ogata, 1917, described filamentous fungus from blood, and claimed to have produced experimental disease in guinea pigs and rabbits with the cultures. Experimentally disease has been transmitted to monkeys, guinea pigs, rabbits, calves, but all Hayashi's cultural experiments negative. The infected animals shewed minute forms similar to those found in man. Biologically the parasite appears to be closely related to Bartonella bacilliforme, and to be a piroplasma=provisionally called Theileria tsutsugamushi, Hayashi, 1920. The transmitting mite is found in the ears of field mice in large numbers, and the mite is only a source of danger in the region of inundated river banks, its bite not producing the disease elsewhere. Deli fever of Sumatra appears to be a fever also transmitted by blood sucking mites.

Note that there is by degrees beginning to be formed an opinion that human piroplasmosis may exist, ? Oroya fever with tick transmission, ? tsutsugamushi fever with mite transmission ? ? blackwater fever with blood sucking insect transmission ? ?

5. *Rocky Mountain spotted fever.* In many ways resembles No. 4. Occurs in Montana and Idaho, U. S. A. Transmitted by tick, Dermacentor andersoni. Ground squirrels, chipmunks, mountain rats etc. constitute reservoir of the virus, and from them infection is transmitted, especially to farmers working in the fields, by the tick. Several classical papers on the disease. Ricketts, 1907, discovered certain bacillus-like forms in circulating blood and in endothelial cells, lanceolate, diplococcal in form or rod shaped. 1 μ long, 1-3rd μ broad, rather resembling influenza bacillus. Fricks obtained ten strains of anaerobic bacilli from infected animals, not pathogenic to guinea pigs, but if cultures inoculated from 21st to 25th day into guinea pigs, lesions caused resembling those of disease.

Wilson and Chowning, 1904, also found piroplasma-like bodies. Ricketts shewed that the virus was not filtrable. Finally came the classical work of Wolbach, 1919. Proves that disease is due to a very minute organism, which he names Dermacentroxenus rickettsi, but which would be better termed Rickettsia dermacentroxenensis, to be in keeping with what follows. Endothelial cells swarm with the infection, being distended by masses of the Rickettsia, see Wolbach's splendid plates. Similar lesions in guinea pig. Exact nature of Rickettsia not known, ? a piroplasma, ? bacterium. Two forms of the organism, (a) rod like, (b) like diplobacillus. Gut epithelium of tick also loaded with the infection. Many workers have tried to shew that the so-called Rickettsia are not parasites at all, but=mitochondria, but recent paper by Nicholson, 1923, proves that they are not. Must be regarded as true parasites.

The tick feeds chiefly on grazing animals, and interesting experiment conducted in 1916 by King, area of 1200 acres taken and 1000 sheep grazed on it, then driven away in hopes that ticks would be cleared with the sheep, failed however to eradicate them. Tick infects via saliva and once infected remains infective for life. Virus can be kept going from guinea pig to guinea pig by weekly passage of blood.

Rocky Mountain spotted fever the first of the Rickettsia infections to be proved as such.

6. *Trench Fever.* Disease of tremendous importance during the war, not so much from its mortality, as from large numbers of men it kept temporarily away from firing line. Due to Rickettsia quintana, so-called from the 5 days of fever. Organisms, like other Rickettsia, very small, 1½ to 2 μ long, ⅓rd to ½ μ broad, Gram negative, stain best with hot Giemsa. Characteristic bipolar staining. The virus is present in the blood, in plasma, and in washed R. B. Cs. Withstands a temperature of 56°C for 20 minutes, but not one of 80°C for 10 minutes. Disease can be artificially transmitted by injection of infected blood, but is in nature transmitted by pediculi. Rickettsia of trench fever thicker and plumper and stain more deeply than do Rickettsia of typhus. In fed louse the Rickettsia are not intracellular, as in tick in Rocky Mountain spotted fever, but remain free in the lumen of the gut. Strong also shewed that man might be infected from contamination of abrasion of skin of hands or—more usually—feet with urine from trench fever case. Louse bite probably not infective, but louse body is and also louse fæces. Usually accepted explanation of epidemics during war is that the sol-

diers were heavily infested with pediculi, scratched themselves vigorously, thus causing abrasions, and if louse's crushed body or lice fæces scratched into abrasion, infection resulted. Louse fæces only become infective seven days after feeding.

Trench fever a second instance of Rickettsia disease, but louse transmission.

7. *Typhus.* Today the most important post-war medical problem of the world. Immense volume of war and post-war literature. In 1922 Bolshevik authorities in Russia admitted to date 9 million deaths from disease since the revolution, and probably many more occurred. Pediculus the proved carrier, Rickettsia the ? proved micro-organism responsible. Numerous commissions during war by different combatant nations on the disease. Recent work by Serbian Red Cross Commission, summarised in Tropical Diseases Bulletin, June 1922, p. 448. They summarise :—

(a). They got healthy lice from non-typhus areas, London stock laboratory and Montreal strains. Dissection of samples shewed no Rickettsia in the healthy lice.

(b). Fed lice in feeding boxes on selected and very early cases of typhus. Early feeds, lice in 27 out of 52 boxes infected ; later, as technique improved, and attention paid to temperature, humidity etc. lice in 18 out of 21 boxes took. On dissecting and examining the fed lice found that infection was limited to their alimentary tract below the oesophagus. Rickettsia bodies found, Rickettsia prowazeki, which were wholly intracellular inside the epithelial cells of mid gut. Gut wall cells distended with the infection in massive degree. Rickettsia bodies thinner and more slender than those of trench fever, ½ to 1 μ long, ¼ to ½ μ broad. Forms met with in lice fed on typhus cases very pleomorphic, bacillary, filamentous, coccoid, diplococcal, etc.

(c). Emulsions made of viscera of infected lice and injected into guinea pigs, one louse per guinea pig. Guinea pigs do not get typhus, but they get fever only to be detected by taking temperatures daily. Incubation period 7 to 18 days, and fever lasts for a week. Every one of these guinea pigs took, some shewed scanty symptoms, were killed, and the histological lesions in their brains and skins found to be identical with those of typhus in man. On recovery the others were now immune to inoculation with typhus blood. Concluded that these guinea pigs had developed true typhus from injection of the infected lice.

(d). Other control guinea pigs injected with control lice from positive boxes, but which, on dissection had shewn no Rickettsia. Of 13 guinea pigs injected from such boxes, 4 developed fever. Of 12 further guinea pigs injected with lice which had never been allowed to feed and which had been kept away from all contact with typhus cases, none took. First lot shewed subsequent immunity against injection of typhus blood, second lot were susceptible.

(e). Careful examination of human lesions in typhus shewed similar Rickettsia bodies in man. Rickettsia swarm in the skin rash in the endothelial cells, in endothelial cells of the typhus nodules in the brain, and in vascular endothelium throughout the body generally. 21 out of 28 specimens of typhus skin rash removed during life shewed Rickettsia. Rickettsia found in brain in 7, testes in 3, kidneys in 2, femoral vein endothelium in 1 case post mortem. Similar Rickettsia findings in infected guinea pigs.

Commission conclude that typhus is due to Rickettsia prowazeki, and louse, as now very fully proved, the transmitting agent. No Rickettsia found in oesophagus or salivary glands of lice : but crushed louse and louse fæces infective. Case for Rickettsia must be taken as almost, if not quite proved. Plotz isolated a Gram positive bacillus from blood of typhus patients, B. typhi exanthematici, but this now of only historical interest. As is well known serum of typhus patients agglutinates certain bacteria of the proteus type, =Weil Felix reaction : chiefly with Bacilli X_2 and X_{19}. Titre at 5th day about 1 in 25 : by second week about 1 in 100. Reaction non-specific but apparently diagnostic of typhus infection.

Loewe and others have cultivated typhus virus, using deep tubes containing 10 c.c. of a rich ascitic fluid and a piece of sterile rabbit kidney. Inoculated with 2 c.c. typhus blood, add 0.3 c.c. of sterile 20% dextrose solution, seal with liquid paraffin : pH 7 to 7.4 : incubate at either room or body temperature. 1 c.c. of a fourth generation culture infected a guinea pig=0.00000016 c.c. of original typhus blood, which indicates that multiplication had occurred in the cultures. The cultures shewed Rickettsia-like bodies, but with hazy outlines, and even more minute than those in typhus lesions.

Impossible to summarise typhus literature here, but whole findings very suggestive. Typhus certainly occurs in India, as Kumaon fever, etc. ? tick transmitted ? louse transmitted. See papers by Megaw and Cragg, I. M. G., 1921-22.

To summarise we here recognise three diseases due to Rickettsia, one with tick transmission, and probably infection of tick progeny : the others with louse transmission. Nature of Rickettsia bodies doubtful, but the diseases shew certain analogies to the relapsing fever group. Position being slowly but surely cleared up by work of many careful investigators, amongst whom for brilliant technique and carefulness Wolbach stands pre-emiment.

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LECTURE 19.

THE SPIROCHAETES (CONTINUED). T. PALLIDUM. T. PERTENUE. S. VINCENTI AND VINCENT'S INFECTION. NAGA SORE AND ULCUS TROPICUM. S. BRONCHIALIS. T. MUCOSUM. SPIROCHAETES OF THE MOUTH, SKIN, INTESTINE, AND MUCOUS MEMBRANES.

The Treponema group or "genus" characterised by:—(a) ends finely pointed, (b) very difficult to stain and culture, (c) pallor when seen under dark ground illumination. (d), regular, even, smooth symmetry, every bit of each spirochaete looking the same, and—except for variations in length—all of them looking the same, monomorphic. Other spirochaetes shew much greater difference in individuals. Treponema smaller than most other spirochaetes.

1. *Treponema pallidum*, vel. Spironema pallida, the parasite of syphilis. Discovered by Hofmann and Schaudinn, 1905. Cultured first in pure state by Noguchi, 1912. The spirochaete is found in :—(a) the primary hard chancre, usually in considerable numbrs. (b) also numerous in the secondary lesions and especially in the roseola. (c) in the lymphatic glands draining the hard chancre. (d) in scanty numbers in the blood and internal viscera. (e) with difficulty in the tertiary lesions, e.g. in the peripheral margins of gummata. (f) with the utmost difficulty and only by special technique, Mott and Noguchi independently, in the cerebral cortex in neuro-syphilis. In congenital syphilis, if the material can only be got fresh, the spirochaetes are found in immense numbers in the liver, spleen, decidua, placental villi, and umbilical cord.

For possible mechanism of transmission vide Lecture 17. The spirochaete of syphilis varies in length from 4 to 20 μ, usually about 8 to 9 μ, or just longer than average diameter of an R. B. C., a useful point in identifying. It has from 6 to 14 small, very even and smooth, tight turns. Under dark ground illumination it is the "pale" spirochaete. Often when seen first is visible as a series of illuminated dots, which suddenly take on spirochaete movement, and true form of spirochaete revealed. Except for length one individual shews little or no variation from another.

Life history of T. pallidum summed up in one word, "unknown." Several workers, amongst others E. H. Ross and McDonagh, misled by Schaudinn's classical muddle of the spirochaetes, trypanosomes and leucocytozoa have put forward most wonderful and weird theories as to its life cycle, identifying it as really a "leucocytozoon" or lymphocytozoon : and meaning really a haemogregarine. All these may safely be ignored. Important work by Levatidi and Marie, 1919, on the different *strains* of T. pallidum. Distinguish between (a) the usual dermatropic strain with special affinity for skin and viscera, and (b) neurotropic strain. Thus (a) is responsible for syphilis as usually seen, e.g. in Algiers severe syphilis of usual type very prevalent, but neuro-syphilis almost unknown. On the other hand (b) the neurotropic strain will cause but little evidence of cutaneous or visceral syphilis, transient chancres which may be overlooked, and makes especially for the C. N. S. It is quite possible that we may in reality be dealing with three almost identical spirochaetes derived from the same ancestor, one causing typical syphilis, a second typical framboesia, and the third neuro-syphilis.

T. pallidum cultivated with some difficulty. Noguchi's technique :—Test tubes 2 by 20 cms. are filled with 15 c.c. of medium consisting of 2 parts of 2% slightly alkaline agar to which, when melted and cooled down to 50°C, add one part of ascitic or hydrocele fluid. At bottom of medium is placed fragment of fresh sterile tissue, e.g. rabbit kidney or testis. After the medium solidifes a layer of sterile paraffin is run on to cover the solid medium to depth of 8 cms. The material, serum from chancre etc., is inoculated into the depths of the medium with a long sterile capillary pipette. The tissue removes oxygen from the medium and gives anaerobic conditions. In a modification he advises the use of two tubes : lower tube as above but after inoculation no paraffin added. Instead take a second smaller test tube : draw out its end into tube : fit through cork which fits the mouth of the lower tube. After inoculating lower tube fill it with medium, push in cork to seal. In upper tube next add second bit of rabbit kidney, and more ascitic fluid. Inoculate again deeply from chancre exudate : cover with 8 cms. layer of paraffin, insert cotton wool plug. Lute the joints of upper and lower tubes with wax. Incubate at 37°C. Growth occurs first in about 9 to 12 days in lower tube as a haze around the bit of tissue, later in upper tube also.

Schereschewsky, 1909, succeeded in getting a mixed culture, but Noguchi was the first to get a pure culture. M'Leod and Soga advise simple modification of Noguchi technique as follows :—Take a test tube and fit a rubber stopper which can be pushed down into the tube. Through the stopper pass a bit of glass tubing, drawn out at its free end into capillary tube, bent over at an acute angle. Test tube is next filled ½ to ⅔rds full with neutral bouillon. This is freshly boiled, and when it is cold a piece of sterile tissue, e.g. rabbit kidney is dropped into it. A strip of sterile gauze is drawn through a sterile glass bead, and soaked in the culture material, e.g. chancre exudate. It is dropped into the bottom of the tube alongside the tissue. Ascitic fluid is then added to nearly fill the tube. As quickly as possible push the rubber stopper down into the tube until the glass tube and its capillary end fill with culture fluid. Seal capillary end and lute all joints with wax. Incubate at 37°C. Material for examination can be obtained by breaking off the capillary tip, and withdrawing from bottom of tube with a long capillary pipette.

Muhlens and Hofmann use serum agar, as follows :—Fill sterile test tubes one third full with horse serum. This is sterilised on three consecutive days at 55°C. Then add equal amount of 3% agar, containing 0.5% glucose which has been melted and cooled down to 50°C. The mixed serum and agar is then kept at 55°C for two hours. Inoculate into depths of medium and incubate under strictly anaerobic conditions.

Or Wenyon's modification of Noguchi medium may be tried : vide ante, Lecture 17.

The best method of securing strictly anaerobic cultures is that introduced in connection with the anaerobes of war wounds by McIntosh and Fildes, 1916, in which the culture medium is enclosed in an airtight vessel which has suspended in it a piece of asbestos wool impregnated with palladium black. The vessel is then attached to a hydrogen generator and hydrogen passed in when it is occluded by the palladium and combines with any oxygen present. A negative presure thus develops inside the vessel and hydrogen will continue to pass in until no oxygen remains. The vessel is then disconnected from the generator, but the action still continues, all traces of oxygen are absorbed and almost perfect anaerobiosis assured.

Method of examination for T. pallidum. Here the worker's success will entirely depend upon the amount of trouble which he takes. It is ABSOLUTELY ESSENTIAL to get the right material, not blood or pus, but the sero-sanious exudate which exudes from the chancre or other lesion investigated. For a hard chancre proceed as follows :—First cleanse with cotton wool swab and get a dry, clean surface. We can then either (a) prick the growing margin of the chancre with a capillary pipette : or (b) prick the growing margin with a needle. Let all bleeding stop and wipe off clot. Get the serous fluid which subsequently exudes. Or (c) rub the sufrace of the chancre after cleaning with the blunt end of a glass side : wait till all bleeding stops, get the serous subsequent exudate and examine this. For examination the dark ground method is to be preferred above all others : but the Indian ink, collargol, Congo red methods or Fontana's stain may be used : see Practical Notes. If Giemsa's stain be employed the best procedure is as follows :—Let the film of serous exdutae on the slide dry thoroughly. Fix with methyl alcohol : (the text books say heat, but this is useless). Wash in distilled water. The slide is now ready for staining. Cover the slide with diluted Giemsa stain and heat. The readiest and least messy way of warming a slide for any staining procedure is to cover the slide with the stain as it lies on a staining rack consisting of two bits of glass tubing fixed on the half of a Petri dish with plasticine : round the end of a wire or stick wrap cotton wool, dip in methylated spirit, set alight and hold under the slide until steam rises from it. Warm till steam rises, about 30 seconds. Pour off and add fresh Giemsa stain. Warm. Repeat 4 or 5 times : staining for ten minutes and finally leaving the stain on for 20 minutes. Wash well with distilled water. Dry without blotting. The spirochates stain rose red. A DIAGNOSIS MADE BY FINDING T. PALLIDUM IN A SYPHILITIC LESION IS WORTH A DOZEN POSITIVE WASSERMANN TESTS. The Wassermann test is merely a test for complement-deviation and is only of value if positive : e.g. a case of a perforating ulcer of the hard palate in a professional prostitute, untreated, and with two consecutive and completely negative Wassermann reactions.

In nature the parasite of syphilis confined to man. Metchnikoff and Roux's classical experiments of 1903, two years before the parasite was discovered. Took material from hard chancres and inoculated female chimpanzees on the vaginal mucosa after scarification. Hard chancres resulted. Incubation period to chancre averaged 30 days : and in further 33 days secondary lesions appeared. Today experimental ape and rabbit strains kept going in many big laboratories. Rabbit easily and readily infected by inoculating juice from hard chancre intratesticularly. Wade Brown and Pearce, 1920-21, have made a full study of experimental syphilis in the rabbit, shewing how strains may be kept alive for years on end : reactions to experimental drugs, serological reactions etc. tested. The bigger the primary sore in rabbits the less likely the disease to become generalised.

In neuro-syphilis in addition to the Wassermann reaction on the blood and C. S. fluid certain useful subsidiary tests : e.g. (a). test the C. S. fluid for increased globulin content. In a narrow test tube to one c.c. of C. S. fluid add on top and carefully one c.c. of filtered saturated solution of ammonium sulphate. Leave for some time. If excess of globulins present a white ring will form at junction of fluids. (b). Count the total leucocytes of the C. S. fluid, noting proportion of lymphocytes. In neuro-syphilis lymphocytosis is present. (c). Colloidal gold test of Lange on C. S. fluid also useful.

Harmless spirochaetes are very frequently present on chancres of all types, and on healthy and diseased mucous membranes : vide below. The normal healthy genitalia often shew numerous harmless spirochaetes : vide an excellent paper by Noguchi, Journal Experimental Medicine, 1918, Vol. XXVII p. 667. S. refringens and S. calligyrum quite common and must be carefully differentiated from T. pallidum.

2. *Treponema pertenue*, the parasite of yaws, discovered by Castellani, 1905. As indicated by name "pertenue" is a very delicate and fine spirochaete. Morphologically it is practically indistinguishable from T. pallidum. Personally believe that there are slight morphological differences : e.g. pertenue more variable in length, shews more tendency to lateral movement, has special tendency to round up into ring forms which pallidum does not shew, perhaps less uniform and more variable than T. pallidum. But any morphological differences are very minor indeed. Size 6 to 20 μ, with uniform, small spiral twists. Ranken saw it shedding granules under dark ground, but ?.

The real case for T. pertenue as different species from T. pallidum rests on the entirely different clinical picture presented by the two diseases. Many essential points of difference. Thus, as compared with syphilis, framboesia (a) is hardly ever or never congenital. (b). the primary sore is almost always extra-genital. (c). secondary lesions rarely or never affect mucous membranes, alopecia unknown, eyes unaffected. (d). tertiary stage often delayed for years, during which only "yaws reminders" are present. (e). C. N. S. never involved. (f). little or no constitutional disturbance. (g). scars much more keloid in yaws, lesions develop more slowly, yaws affects skin tissues much more and internal viscera much less than does syphilis. Yaws responds to both mecury and salvarsan more readily than syphilis. Consider that most workers agree that yaws is not "stone age syphilis" as Parham and Bulter think, 1922.

These writers insist on identity of syphilis and yaws. Quote Samoa as example. No syphilis known in Samoa although the island has been in contact with European sailors for 200 years. Syphilis unknown in Samoa, yet almost every inhabitant gets yaws. They conclude that yaws is syphilis of savages, of directly contagious, infective type, and gives immunity against syphilis. State that in Samoa syphilis = the commonest exanthem of childhood and protects against syphilis in adults.

Curious fact that parasites of syphilis and yaws should have been discovered in same year, 1905. T. pertenue must be present in the blood, although not demonstrated by microscopic examination, since monkeys injected with blood of yaws cases get infected, although no spirochaetes can be found. Charlouis, 1881, inoculated a native suffering from typical yaws with syphilis: typical hard chancre developed at site of inoculation. Many instances of the development of yaws, both naturally and by inoculation, in those affected with syphilis. Levatidi and Nattan Larrier have noted that monkeys inoculated with syphilis are immune to yaws: but yaws monkeys could be infected with syphilis. According to Ashburn and Craig monkeys immunised to T. pallidum are still susceptible to T. pertenue and vice versa.

T. pertenue found in primary yaws chancre. Also in spleen, lymph glands, bone marrow, and secondary lesions. Technique for examination :— best is to scrub the surface of the yaws lesion with blunt end of glass slide until it bleeds, mop off blood, get serous exudate that follows. Yaws lesions often fail to shew T. pertenue unless this is done, as recent experience, 1923, at Calcutta has shewn. Also may get S. refringens etc. from surface and not T. pertenue at all. T. pertenue so far never found in C. N. S. or C. S. fluid.

In sections of skin lesions T. pertenue found in interpapillary plugs of Malpighian layer of epidermis, whereas in syphilitic lesions T. pallidum found especially in the thickened arteries in the corium.

Paulet, 1848, inoculated 48 negroes with exudate from yaws lesions and all developed the disease, incubation periods 12 to

20 days. Charlouis, 1881, inoculated 32 Chinese prisoners with yaws crusts, 28 contracted the disease and primary lesion in each case appeared at site of inoculation. Yaws is easily transmissible, and much more so to animals than is syphilis. In higher apes the infection generalises, in lower apes remains as primary lesion only, for 16 to 92 days. Secondary lesions may or may not appear. A most careful study of T. pertenue as seen in several dozens of positive dark ground films from recent collection of yaws cases at Calcutta shews that division of the spirochaete is always by transverse division, process having been seen under dark ground. Y forms are quite common. Blood from spleen puncture of yaws cases has always given negative results : Knowles, 1923, examined spleen juice from nine cases, with negative dark ground findings, but inoculated monkeys still take.

Castellani sums up evidence in favour of T. pertenue as true parasitic micro-organism of yaws as follows :— (a). Is the only parasite found constantly in non-ulcerated lesions. (b). is also found in lymph glands, and in infected experimental monkeys. (c). spleen puncture fluid, although negative microscopically, is still infective. (d). if the spirochaete be filtered out the fluid is now not infective to experimental animals. Nichols has made full experimental study of yaws : infected rabbits with both yaws and syphilis and studied results of salvarsan treatment in both.

T. pertenue cultured by Noguchi. Appearance in cultures like that of T. pallidum. Nichols first infected rabbits by intratesticular injection. In nature yaws spreads by direct contact., e.g. instance in small village in Kuki Hills, Assam, 1923 : 100 villagers of whom 33 infected. Virus can be experimentally carried by flies, but direct contact usual mode of infection, e.g. mother infected on nipple by infected infant. Quite possible that in Assam and Burma where very prevalent, non-biting flies, Siphonella etc., passively may carry infection. Disease especially prevalent in Java, where majority of natives get it sooner or later in life, in some districts of Burma and Assam, and in Ceylon, where official records report over 3,000 cases treated annually.

The disease known as gangosa is probably only a tertiary form of yaws. The salvarsan derivatives even more effective in yaws than in syphilis. Wassermann reaction usually strongly positive, but of 12 cases, 1923, at School only 5 gave positive Wassermanns, whereas 10 shewed T. pertenue on dark ground examination.

Vincent's spirochete, S. vincenti. Vincent's infection, as it is best called, one of the most interesting in the tropics. Causes at least four, possibly more, conditions: viz:—(a). Vincent's disease of the throat, and other mucous membranes, e.g. of genitalia and ? ? of bronchi. (b). Ulcus tropicum. (c). Naga or N. E. Frontier sore, or Cachar boil. (d). recent case in U. S. A. where it set in as secondary and fatal septicæmia in appendicostomy wound. Also (e). may supervene in colon mucosa as sequel to amoebic or other form of dysentery, and (f). may be identical with Castellani's S. bronchialis, as contended by Vincent himself, 1922.

Extraordinary etiology of Vincent's infection: term Vincent's "angina" should now be discarded since it does not always cause angina in the throat and may affect many other tissues, including even bone. The spirochaete lives in symbiotic relationship—apparently—with the fusiform bacillus. Latter has been cultivated by several observers, the former not so far. Spirochaetes 10 to 20 μ long, with open coarse coils. Rather delicate and slender. Bacillus fusiformis incurved, finely pointed ends, and shewing prominently—especially in young bacilli—metachromatic granules by Giemsa's stain. Motile ? ?. Many other harmless commensal spirochaetes met with in the above mentioned lesions but have not the same association with the fusiform bacillus as here described.

Typically Vincent's infection causes Vincent's angina of the throat: and to some extent doubtful whether Vincent's infection is ever primary, and not rather—whenever it occurs—secondary on some pre-existing lesion. But from Vincent's angina it directly affects other mucosae, e.g. clear cases of penis infected from throat, and possibly=Castellani's S. bronchialis by direct extension to bronchi.

Recent important work by E. C. R. Fox, at Shillong Pasteur Institute 1921, on true etiology of *Naga sore.* Epidemics in Assam Valley on tea gardens and mines, especially during monsoon months, affect labour force often by thousands, laid aside by sickness for weeks on end. Great economic importance of the disease in industries in tropics. Ulcer always starts as accidental abrasion: thus 18 cases followed at site of anticholera inoculation on a tea garden from dirty needle: usually abrasion on ankle, dorsum of foot, or leg below knee: one clear instance in European set in on site of spear grass abrasion. There follows a terrible sloughing ulcer with characteristic foetid odour eroding everything it meets; tendons, fasciae, even bone. Have seen one case fatal from septicæmia, whilst Abraham in Assam records several cases where amputation necessary to save life. Patient usually laid up for six weeks or more. Some Assam tea gardens in 1918 epidemic had over 900 coolies laid up for months together simultaneously. Recent example on Witerwatersraand, 2000 working days lost by 13 infected coolies. In Assam, epidemics all down the Assam Valley are associated with special prevalence during these months of non-biting Siphonella flies. Hall Wright at Mariani fed these flies on a Naga sore, then on abrasion on his own arm, got a much worse Naga sore than he wanted to, with typical bacteriological findings.

Animals practically insusceptible. Fox finally tied dressings soaked in discharge from sore on to abrasions on arms of volunteers : left to dry in situ. Three days later slight ulcer followed, shewed only serous discharge with septic cocci. A few days later Vincent's spirochaete discovered in large numbers, a few days later still the fusiform bacillus in profusion. At this stage films from the developing sores presented exactly the same picture as those from an acute Vincent's angina of the throat. Later on the spirochaetes disappeared, and only fusiform bacilli left, with secondary organisms. Using technique adopted by Semple and his co-workers for Vincent's angina, Fox got the fusiform bacillus in pure culture. Method is to inoculate serum agar whilst still liquid, and fill upper half of Petri dish with inoculated medium. Then to add more medium and invert over it lower half of Petri dish so that anaerobic culture in depths of medium results : Petri dish being sealed with vaselin. The colonies have fuzzy edge, darker centre, cloudy margin, greenish blue colour and exactly the same fœtid odour as the original Naga sore. Spirochaete not yet cultivated.

Ulcus tropicum has practically the same etiology and is in all probablity Vincent's infection also. Veld sore of S. Africa is something entirely different, ? diphtheroid infection. Acton, 1922, at School had a case which began as inguinal soft sore due to du Crey's bacillus, then shewed invasion by fungi : later half healed, then got Vincent's infection and, broke down again to present typical appearance and microscopical picture of Ulcus tropicum. Believe that Vincent's disease may affect (a) the throat as Vincent's angina : (b) other mucous membranes such as genital, gut, bronchi : (c). subcutaneous tissues as in Ulcus tropicaum and Naga sore : and may—possibly—even cause septicæmia. Please be on most careful look out for Vincent's infection : e.g. grass cutters' and syces' ulcers on shins in cavalry regiments during rains in the

U. P. and Punjab may possibly be the same, as clinically identical.

S. bronchialis, Castellani, 1907 is stated to be cause of Castellani's bronchitis, which greatly simulates phthisis with symptoms of fever, cough, haemoptysis etc. Both acute, febrile and chronic, non-febrile types of the disease recognised. Sputum is often loaded with spirochaetes and is frothy, bright red, with much fresh blood. Spirochaetes said to be very pleomorphic, 5 to 25 µ long, and varying in thickness. Very highly motile in fresh state. Fantham stated that he saw this spirochæte shedding granules under dark ground illumination and belives infection disseminated by such granules in desicated sputum. Case for Castellani's spirochæte as sole cause of special form of broncho-pneumonia fairly good, but far from proved. Vincent, 1922, considers that Castellani's spirochætal bronchitis = Vincent's infection merely of bronchi, (and is probably right). The spirochætes are said to be found in immense numbers in the blood stained sputum, which is alive with them, whilst X ray photographs shew no evidence of pulmonary phthisis.

Passing from these clearly identified and—at least mostly pathogenic—spirochaetes we come to enormous numbers of human spirochaetes where the pathogenicity is at least dubious or obviously nil. As follows :—

S. refringens. An exceedingly common spirochæte on skin lesions and on both healthy and diseased mucous membranes, as also probably in the gut. Important not to give a mistaken diagnosis of syphilis on the finding of S. refringens in a soft chancre. Called " refringens" from its bright, white, glistening appearance under dark ground illumination. Is a big, thick, heavy type of spirochæte with larger, coarser, more open coils by far than T. pallidum. Stains readily with any aniline dye. Many harmless spirochætes may be encountered in either pathogenic or non-pathogenic lesions of the skin and mucous membranes :. Thus :—S. aboriginale, 6 to 17 µ, encountered in cases of inguinal granuloma from Guam. S. phagadenia ? = S. vincenti, described from indurated swelling of labium major. S. acuminata and S. obtusa from yaws ulcerated lesions, Castellani, and have personally seen S. refringens in several yaws ulcers. S. pseudo-pallida from open surface of carcinoma ulcers, Mulzer. These are admittedly harmless and non-pathogenic.

With regard to others we have :—S. minuta, Castellani, 1916 from cases of rhinitis, small open spirochaete with open coils, 8 to 12 μ. S. urethræ Macfie, 1917, in pus from cases of non-specific urethritis, said to be both extra and intracellular.

Spirochætes are very common in fæces *if looked for*. Calcutta 1922 figures give numerous spirochætes seen in 42 out of 730 stools. The one usually met with is *S. eugyrata*, so-called from its large, smooth, even symmetry. Discovered by Warner, 1909, in stools of dysentery convalescent. Macfie found it in fæces of diarrhœa case on Gold Coast. Movements very regular and coils open and very symmetrical, but much bigger than a Treponema. Vincent's infection not at all infrequently met with as sequel to dysentery of any type, and as transient infection of gut, (Acton, Knowles). Often find one day the stool is loaded with spirochætes, next day or two days later none. Insects and animals harbour any number of spirochætes in gut, almost every cockroach has small stumpy spirochæte, with 2½ to 3 turns, actively motile, and similar species found in gut of frogs and rats and lion.

In the mouth we get many spirochætes. S. re-fringens is very common and harmless. In syphilis we may get T. pallidum, and in Vincent's angina S. vincenti. Noguchi identifies two common dental and oral spirochætes. (a). *S. dentium:* vel. S. buccalis, small, rather pale spirochæte, not unlike T. pallidum but thinner and more variable. (b). *S. vel Treponema mucosum*, Noguchi, 1912. 6 to 12 μ long, 6 to 8 curves, thicker than other Treponemata. In Noguchi culture gives rise to abundant mucinogen. On injection of culture into animals hypodermically causes much marked local œdema and infiltration. Cultures have same peculiar fœtid odour as pyorrhœa case. Noguchi considers that this spirochaete may possibly be of importance in pyorrhœa. But other workers find both S. dentium and T. mucosum in healthy gums. Claim for spirochaetal causation of pyorrhœa alveolaris much better than claim for entamœbæ (or streptococci), but whole etiology of the disease—a most important Indian medical research problem—remains to be worked out.

Animal world swarms with natural spirochaetes. S. muris of rats produces fatal epidemics of relapsing fever type. Rats are infected with any number of spirochaetes, especially met with in blood, kidney smears and urine, which may—or may not—be important in connection with human diseases. S. ovis of sheep and S. theileri of cattle apparently non-pathogenic, inhabit blood. Two excellent and favourite examples for laboratory study are S. anserina of geese and S. gallinarum of fowls which cause severe relapsing type of fever, often fatal, in geese and fowls. In S. gallinarum infection early on S. gallinarum swarms in peripheral blood of infected fowls, 9 to 12 μ with rather open coils. Blood periodically clears and then spirochætes re-appear as with all relapsing fevers. As convalescence sets in long forms appear, 16 to 19 μ, and still later end to end forms of very great length. At periods of absence of spirochætes from peripheral blood chromatinic granules seen in cytoplasm of R. B. Cs., stain deeply, ? capable of growing up into new spirochætes, Balfour. S. gallinarum favourite object for laboratory study. Hereditary transmission via egg to chicken said to occur.

Practical Notes.

Treponemata are difficult to stain. The hot Giemsa method detailed above will be found useful. The usual stain for films is, however,—*Fontana's stain.* The film of serous exudate from the chancre must first be allowed to dry absolutely. 2. Fix for one minute with methyl alcohol : this is essential, although omitted in text books : it greatly improves the chances of a positive finding. 3. Wash in distilled water. 4. Lay slide on staining rack and flood three times with Huge's solution :=glacial acetic acid, 1 c.c. : formalin 20 c.c. : distilled water to 100 c.c. 5. Wash in tap water, and soak in following mordant—carbolic acid 1 c.c. : tannic acid 5 gm.: distilled water 100 c.c. This mordant is much improved, and the stain free from deposit if the tannic acid used be only the ether-soluble fraction. (To prepare dissolve tannic acid in ether, and filter. Evaporate the ether off from the filtrate and use in place of the tannic acid above).

6. With the slide covered with the mordant heat till steam rises. Allow to cool for 30 seconds. 7. Wash in tap water. 8. The slide is now mordanted and ready to stain.

9. To prepare the silver solution :—In a test tube place 5 c.c. of one-quarter per cent. silver nitrate solution in distilled water. Add drop by drop to it liquor ammoniae until a faint whitish opalescence occurs. At this point stop, and do not add any more ammonia. 10. Place the slide on the staining rack, flood with the prepared silver solution, and heat till steam rises. Allow to cool, 30 seconds. Wash in tap water. Dry. Examine with oil immersion lens.

The silver deposits in the spirochaetes and oxidises, so that they appear brown-black against a pale yellow background. Fontana's is quite the best stain for Treponemata, but Fontana stained slides fade on keeping very rapidly.

Levatidi's Stain for Treponema in Tissues.

This is a splendid stain for Treponema if probably applied.

1. Material for Treponema must always be fixed in formalin. Put pieces of the tissue, e.g. liver of congenital syphilis, not more than one mm. thick in 10% formalin in saline for 24 hours.

2. Rinse in water and soak for 24 hours in 95% alcohol.

3. Place in distilled water until the tissue sinks to the bottom of the container.

4. Place in 2% silver nitrate solution and keep in the 37°C incubator for four or five days.

5. Wash in distilled water and place for 24 to 72 hours in following solution. Pyrogallic acid 3 gms. : formalin 5 c.c. : distilled water 100 c.c.

6. Wash in distilled water.

7. Pass up through 30%, 50%, 70%, rectified and absolute alcohols very rapidly, (otherwise the stain fades), into mixture of equal parts xylol and absolute alcohol. Soak in this. Transfer to xylol pure.

8. Embed in paraffin. Section. Mount sections on slides in usual manner. Remove paraffin by xylol. Mount in balsam, which should be neutral and non-acid.

Levatidi's stain shews the spirochaetes stained intensely black, the reticulum of the tissues stains brown, and other tissue elements yellow. No counter stain should be used, as counter staining only renders the spirochaetes more difficult to see.

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LECTURE 20.

THE CHLAMYDOZOA. TRACHOMA AND CARCINOMA BODIES. THE NEGRI BODY OF RABIES. SOME NOTES ON RABIES. CELL-INCLUSIONS. THE KURLOFF BODY.

The "Order" Chlamydozoa created by Prowazek, 1907, for a group of supposed living organisms with following characters :—(a). all have ultra-minute phase in life cycle. (b). virus concerned is in each instance filtrable. (c). If, however, the filter be coated with agar the virus does not pass through. (d). all live in the cytoplasm of either epithelial or nerve cells : (note that embryologically nerve cells = epiblast). (e). Each is supposed to have a rudimentary life cycle of similar character. The name Chlamydozoa derived from chlamyx = a veil, and zoon = living animal, and the Chlamydozoa are " veiled protozoa" in more senses than one.

Most important members of group are :—(a). Negri body of rabies. (b). the Guarnari bodies in the epithelial cells in vaccinia and variola. (c). Prowazek's bodies in epithelial cells of conjunctiva in trachoma. (d). Mallory's bodies in desquamating epithelial cells in scarlet fever. (e). Bodies in epithelial cells in molluscum contagiosum. (f). Bodies in carcinomata cells, cancer bodies.

Prowazek and Hartmann describe life cycle of a Chlamydozoon as follows :—Begins as ultra-minute particle, the so-called infective granule. Infective granule grows to diameter of 2 to 6 μ. As it does so there occurs around it a cytoplasmic reaction, depositing a sort of veil around it, whence the name Chlamydozoon. Some workers consider that the mantle is thrown out en masse from the host cell nucleus. Inside this mantle the parasite divides up into an immense number of tiny ultra-minute infective granules = schizogony. Finally the infected host cell breaks down, setting free broods of infective granules which enter fresh cells to repeat the life cycle. Parasitic theory of cancer is largely due to chlamydozoal hypothesis.

Chief points of evidence in favour of view that the Chlamydozoa are living organisms as follows :—(a). Noguchi and Cohen, 1913, claim to have grown the virus of trachoma in artificial culture. Their cultures however failed to produce trachoma in experimental monkeys. (b). Noguchi also claimed to have cultured the virus of rabies in artifical media, but experiments with no control culture tubes of healthy brain tissue to compare differences, and a most strenuous effort by Acton and Knowles at the Kasauli Pasteur Institute to repeat and confirm Noguchi's experiments resulted in entire failure. The appearances which he describes in his "cultures" as infective granules appear to be merely disintegrating nerve cells etc. (c). Work of Heyman and others on cell-inclusion blenorrhoea. This is a disease of infants' eyes, said to be of non-gonorrhoeal origin. Morbid histology and cell-inclusion masses in conjunctival epithelium said to be exactly like the picture in trachoma. Linder claimed to have transmitted the disease to monkeys by rubbing in pus into conjunctiva from eyes of infected children. Doubtful whether he did not produce septic conjunctivitis merely in the monkeys ?

In connection with these diseases must admit certain fundamental facts. (a). All are due to filtrable viruses, which must be of the nature of particulate living matter. The real nature of these viruses appears to be as "globoid bodies" of almost invisible size, similar to those described by Gordon, 1922, as true causal "micro-micro-organism" responsible for epidemic influenza and for nasal catarrh. Those interested in vaccinia should read most careful paper by MacCallum and Oppenheimer, 1922, on subject. They studied specific gravity of vaccinia virus, found it to be about 1.12 or 1.13 by differential centrifugalisation. Having thus isolated the virus in concentration proceeded to study its characters : shews up microscopically as myriads of tiny granules, much more minute than any coccus. Occur in groups, clusters, chains, and very bright and refractile under dark ground. Are Gram negative, stain blue or red with Romanowsky stains, and fairly deeply with carbol fuchsine. Control preparations from sediments of other specific gravities failed to shew these minute particles. The "infective granule" then must be a reality. (b). Doubtful however whether there exists any so-called cell inclusion blenorrhoea which is non-gonorrhoeal in origin. Also scarlet fever almost certainly due, not to filtrable virus, but to haemolytic streptococci.

Personally consider whole case for the Chlamydozoa as living organisms entirely non-proven. Regard them as being

almost certainly non-living cell-inclusions, and not protozoa at all. Cell-inclusions are very common in nature, and well exemplified by secretory cells of gastric mucosa or secretory cells of venom glands of snakes. Here the pre-zymogen commences in the form of the discharge from the nucleus, often from nucleolus, of tiny chromatin-staining particles into the cytoplasm of cell, where cytoplasmic reaction occurs and they are elaborated into pre-zymogens and excreted from cell.

The Negri body of rabies is certainly not a living structure at all. Most careful study of it by Acton and Harvey, 1911-13, shews its true character. Virus of rabies in nature transmitted only through bites and via saliva. Yet virus may often be absent from saliva of truly rabid animal. Virus when implanted in bite gets to nerve endings and travels up nerves to C. N. S., thence from C. N. S. by efferent nerves to salivary glands etc. C. N. S. always full of the virus, medulla especially, although here the Negri bodies are so small as to be almost invisible.

Procedure in sending up brain of an animal suspected of rabies to a Pasteur institute. 1. Put on pair of leather or thick rubber gloves : this may save a personal and subsequent visit to the Institute. 2. Wash body of dog with antiseptic to kill ecto-parasites and remove infective saliva etc. 3. Lay dog's body on floor and with heavy hammer break up the skull cap. 4. Lay on table. Incise skin antero-posteriorly over top of skull, reflect flaps to each side and expose broken up skull. 5. Remove skull, incise dura mater and expose the brain. Take out the entire brain, by carefully lifting it out, severing the nerves at the base.

Next procedure depends on size of the dog. If brain a small one place entire brain in fixative. If brain a big one, lay on table, remove horizontal slices until lateral ventricles are opened. Open descending horn of lateral ventricle. On its external aspect will be found the eminence of the Hippocampus major. Carefully remove the Hippocampus major of one or both sides. Take wide mouthed bottle. At bottom place layer of cotton wool to prevent shaking. Fill with fixative. Ten times as much fixative as brain should be used. Best is Zenker's fluid : corrosive sublimate, 5 gms : potass. bichromate 2.5 gm. : sod. sulphate 1 gm : glacial acetic acid, 5 c.c. : water 100 c.c. If Zenker not available use rectified spirit. Formalin a very bad fixative for this purpose, distorts and ruins cell picture. Insert brain or dissected out hippocampus. Seal and label,—latter point often forgotten. With parcel send full details of animal's history. But note that the keeping of a dog tied up and under observation for ten days from onset of first suspicious symptoms worth a dozen examinations for Negri bodies in diagnosis.

C. N. S. changes in rabies are 1. acute round celled infiltration in medulla and ganglia generally, including lateral ganglia. 2. Negri bodies : these are best of all seen in the dog in the stellate nerve cells of the Hippocampus major, which is supposed to be organ of smell in dog, and contains biggest nerve cells of its C. N. S. Negri bodies occur in other nerve cells, but not so big and conspicuous. In hydrophobia in man Negri bodies not found. Here, instead, the chief changes occur in the big pyramidal cells of the cerebral cortex, where the poisoned nuclei throw out into cytoplasm of the cell immense volumes of nuclear matter en masse : appearances different to those of Negri body, but etiology similar.

Pasteur applied the term " street virus" to virus of rabies as it occurs in the saliva of the rabid dog of the street. Has a very varying incubation period, either subcutaneously when implanted on nerve terminal, or when given on to surface of brain direct. Pasteur took emulsion of medulla of rabid street dog=street virus, and inoculated by trephining on to the surface of the brain of a healthy rabbit. When this is done the rabbit develops dumb rabies and dies. Disease always of the dumb type in rabbits : have only once seen furious rabies in the rabbit in nearly seven years of Pasteur Institute work. Incubation period may vary from 14 to 90 days. When this rabbit was fully paralysed and its C. N. S. full of virus, Pasteur made emulsion of its medulla and passaged subdurally into second rabbit : thence into third, fourth, fifth, etc. After varying number of passages the virus becomes fixed and of maximal. exalted virulence for growth on rabbit brain= fixed virus of Pasteur. Thus and most ingeniously he obtained a virus of fixed and standardised virulence, although working with an unknown and today still undiscovered micro-organism. In fixed virus rabbit sequelae always the same : front legs paralysed about 6th day, hind legs about 7th day, animal fully paralysed 8th or 9th day, dies 9th day.

All antirabic vaccines in use over the whole world derived from such fixed virus rabbits. Indian fixed virus was first elaborated at Kasauli from street virus from a rabid jackal's brain. Pasteur immunised with desiccated spinal cords of fixed virus rabbits. Allowed the rabbit to die paralysed, removed spinal cord, hung it up in sterile two-way bottle over stick caustic potash. The cords kept in a dark room at 72°F. As cords desiccated virus gradually dies out in them, method is really a rough dilution one. When patient arrived bitten he was given first day one cm. length of 14 day cord= cord desicated for 14 days, and avirulent, emulsified in saline and injected hypodermically. Subsequent injections 13 and 12 day cords : then 11, 10, 9, 8, 7, 6 5, 4, 3, and 2 day cords, an increasingly virulent series. This method in use today at most French institutes and in America. Used at Kasauli from 1900 to 1907, but is a bad method. Objections : prevalent sepsis, risk of hydrophobia from injection of virulent antigen, risk of neuro-paralytic accidents from injection of large amounts of nerve substance into exceptionally susceptible persons. From 1907 to 1911 use at Kasauli and other Indian institutes of Höghyes' dilution method. Here fixed virus rabbit is killed by air embolism when fully paralysed. Medulla=most virulent portion only, of brain taken. Emulsified fresh in saline in 1 in 100 dilution. From this series of dilutions of fresh virus prepared. When patient arrived he got dose of 1 in 4000 dilution, then subsequently 1 in 2000, 1000, 750, 500 etc. down to 1 in 100. Höghyes' method great improvement on dry cords, little sepsis, amount of nerve substance used minimal, but—largely hypothetical—risk of hydrophobia from use throughout of living virus in fresh state.

From 1912 to date Indian institutes now all use Semple's carbolised vaccine. Fixed virus rabbit killed on 9th day when fully paralysed. Whole brain removed, and pounded in sterile pestle and mortar and made into 4% emulsion by weight in ½% carbolised saline. Incubated at 37°C for 24 hours : the carbolic kills the rabies virus and antigen is a dead one. Next day diluted. with equal volume of saline : now= 2% emulsion in ½% carbolised saline. At this stage bottled and stored in sterile rubber capped flasks and three tests applied : (a). aerobic culture to ensure absence of septic organisms. (b). anaerobic culture to ensure absence of tetanus, —a rule in all government institutes. (c). rabbit test : one c.c. injected intranuchally to reach nerves emerging from cervical spine. Rabbit watched 14 days : if shews no symptoms and remains fit we know that the carbolic has truly killed the rabies virus. Any vaccine which does not pass tests discarded. On day vaccine is brought into use it is again diluted with equal volume of saline, and reaches patient as 1% emulsion of dead fixed virus brain in ½% carbolised saline. Patient gets one c.c. hypodermically on each side of abdomen daily for 14 days.

Despite historical and splendid work of Pasteur on rabies, today the value of antirabic immunisation very much doubted.

See papers by Harvey and Acton, 1922-23 : some degree of immunity undoubtedly conferred but very doubtful how much. No advance possible until the real micro-organism of rabies is discovered : one of the most pressing medical research problems of the day.

Acton and Harvey took street virus of dog and fixed it, not for rabbit, but for monkey, Macacus rhesus, by serial sub-dural passages. Studied Negri bodies : at first were large and prominent, but as virus become more and more fixed and exalted, Negri bodies became smaller and smaller and finally invisible. None are seen either in fixed virus rabbits. Finally shewed that Negri bodies are also to be seen under other conditions. e.g. in brains of guinea pigs poisoned by viper venom. Even small ones seen in dogs which die of starvation and in very young healthy puppies, where C. N. S. cells have not yet become stabilised.

True etiology of the Negri body of rabies is as follows :— C. N. S. cells become poisoned by rabies virus. First change is nucleolar degeneration. Nucleolus commences to undergo fragmentation. By degrees these chromatin particles are extruded through nuclear membrane and come to lie in the cytoplasm of nerve cell. Here a cytoplasmic reaction takes place around them, producing the "chlamydozoal" appearance. Appearance of Negri body all depends on how long the dog lives after symptoms set in. If dog dies very rapidly Negri bodies have not had time to form : sole changes seen are very early fragmentation of the nucleolus. If dog lives a few days big and prominent Negri bodies seen. They should be stained by iron haematoxylin. Always use iron haematoxylin, not Mann's or Giemsa's or other stains as recommended by books. The early Negri body very minute and stains jet black. The later Negri body lies (a) outside the nuclear membrane, but (b) in the cytoplasm of the nerve cell, and (c) its staining reaction will depend upon its age. If very young and small stains jet black : if later takes intermediate stain between eosin of cytoplasm and black of chromatin, and brown to pink. Finally if dog lives several days Negri bodies very big, often appear as if vacuolated, and may be found even outside the breaking down nerve cell or even in axon away from cell nucleus. Thus the examination of brain of suspected animal for Negri bodies in rabies is a test only of value if results are positive. If Negri bodies seen in numbers dog had rabies, as large numbers of Negri bodies are characteristic and pathognomonic of the disease. But if none seen, cannot say the dog did not have rabies. Far better test is to place suspected dog under control and observation : if alive and well ten days after onset of symptoms has not got rabies : rabies invariably fatal within ten, more usually within two to five days of onset of first suspicious symptoms.

Amusing statement in 1923 edition of Daniels and Newham's "Laboratory Studies in Tropical Medicine," p. 228, that nothing is known of the development or life history of Negri bodies. They are cell-inclusion phenomena in nerve cells poisoned by the rabies toxin. In hydrophobia in man the poisoned cells of the cerebral cortex react in the same way, only instead of the nuclear chromatin being shot out in particles, it is extruded in bulk into the cytoplasm of the cell, thus producing the "yellow masses" characteristic of the diseased cells.

Acton and Harvey's work on Negri bodies tends to destroy the whole case for regarding the Chlamydozoa as living parasites. In variola and vaccinia we have epithelial cells dying and degenerating : under such conditions what more likely than that chromatin should be extruded into cytoplasm, and with cytoplasmic reaction=Guarnari bodies? In trachoma we have irregular proliferation of epithelium with again every possiblity of extrusion of chromatin from nucleus into cytoplasm. In carcinoma we have epithelial carcinoma cells which have acquired a terrific and uncontrolled rate of multiplication. All sorts of erratic and aberrant phases of nuclear division, true mitosis, amitois, irregular mitosis etc. Under such conditions what more likely than that in these rapidly and erratically dividing cells bits of waste or aberrant chromatin get left behind in the cytoplasm during cell division, cytoplasmic reaction takes place=carcinoma body? Personally regard the whole case for the Chlamydozoa as living parasites as non-proven until better evidence is put forward.

The *Kurloff body* of the guinea pig another interesting case of a cell-inclusion. Subject of wild speculation for years. E. H. Ross describes it as a parasite of the lymphocytes of the guinea pig, "Lymphocytozoon cobayae" and "grew" it on special jelly medium. Kurloff bodies are found in peripheral blood of something like 40% of guinea pigs, especially of young guinea pigs. But also seen in bone marrow films from pigeons, and many different types of animals : as anyone who does work for a Zoo will soon discover.

In peripheral blood the Kurloff bodies are met with in the cytoplasm of certain leucocytes which resemble, but are not, large hyaline mononuclears. As shewn by Acton and Knowles, 1918, their appearance depends absolutely on the fixatives used for the films, and it is this which has misled workers into describing their "life cycle." If fixed and stained by Leishman all sorts of bizarre appearances seen which may be misinterpreted into a dozen different life cycles. But if wet fixation with Schaudinn's fluid be employed and iron haematoxylin staining their appearance is quite uniform, they shew a definite and marked outline, and a hyaline non-staining interior. In reality as shewn by Acton and Knowles, 1914, the Kurloff body is simply a stage in the development of the C. G. eosinophile leucocyte. All phases of its development may be seen in bone marrow films. This leucocyte begins with large single nucleus and pale blue staining cytoplasm, with Romanowsky stains. From the nucleus is extruded into the cytoplasm an "initial korper" or chromatin mass. This grows bigger and bigger. As seen in peripheral blood films it=the Kurloff body. By degrees there forms within it a spireme thread, which then splits into two longitudinally. The whole structure gets larger and larger, coming to almost completely fill the cell, and compressing the cell nucleus at first into a horse-shoe shape, later almost into two halves. By degrees the spireme thread breaks up into the coarse granules which take a deep red stain with eosin, and the cell becomes a C. G. eosinophile of the usual pattern.

In brief cell-inclusions very common in nature and are objects well worthy of further study.

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